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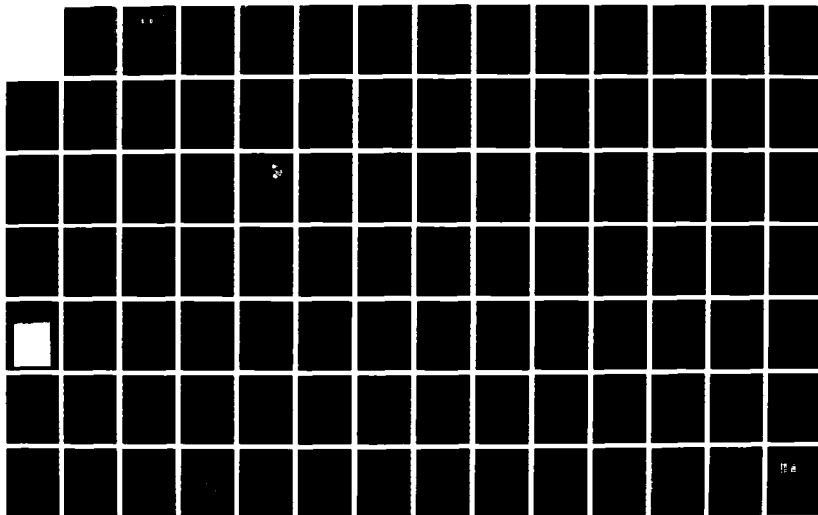
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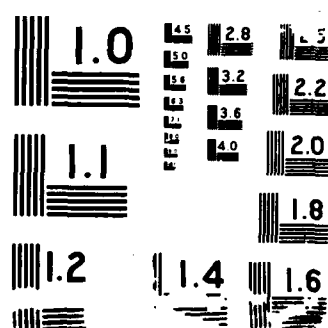
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→ The finding that relatively large current densities were required to measurably increase protein synthesis does not necessarily indicate that physiological current densities are unimportant.

The change in total protein synthesis indicates that electric fields may play a role in physiological regulation of chondrocyte metabolism. These results may have important implications regarding clinical applications of fields to alter growth and remodeling. ←



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Cartilage:  
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by

**Laura A. MacGinitie**

S.M., Massachusetts Institute of Technology, 1982

S.B., Massachusetts Institute of Technology, 1980

Submitted in Partial Fulfillment of the  
Requirements for the Degree of  
Doctor of Philosophy

at the

**Massachusetts Institute of Technology  
December 1987**

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Department of Electrical Engineering and Computer Science  
December 1987

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# **Electrical and Thermal Modulation of Protein Synthesis in Cartilage: A Model for Field Effects on Biological Tissues**

by

**Laura A. MacGinitie**

Submitted to the Department of Electrical Engineering and Computer Science in partial fulfillment of the requirements for the Degree of Doctor of Philosophy,  
January 1988

## **Abstract**

Interest in the effects of electric fields on biological tissues has been motivated by the natural occurrence of fields within tissues, which suggests that they may play a role in physiological function. The possibility that applied electric fields may alter physiological function, either in a beneficial or harmful way, has raised further significant interest.

In this thesis, the issue of the effect of electric fields on biological tissues was addressed using field-induced changes in stress protein and total protein synthesis in cartilage tissue as an experimental model. In cartilage, electric fields and currents are generated during physiological loading. The amplitudes and frequencies of the applied fields were motivated by the values associated with the naturally occurring fields, and by some models of possible mechanisms of interaction. Synthesis of stress proteins is induced by a variety of toxic agents. Therefore, it is an appropriate marker for stress induced by applied fields over a wide range of frequencies, in which several mechanisms of interaction might be important. Total protein synthesis serves as a marker for a generalized field-induced change in cellular metabolism.

Culture conditions for the cartilage tissue were chosen with a view to optimizing the sensitivity of stress and total protein synthesis to a stimulus such as the electric field, and to produce steady state protein synthesis prior to electrical stimulation. The response to heat, a stimulus known to produce a stress response was used to examine the sensitivity of cartilage to a stimulus under a variety of culture conditions—different serum concentrations and days of culture.

Cartilage specimens were exposed to current densities up to 30 mA/cm<sup>2</sup>, at frequencies of 1, 10, and 100 Hz and 1 and 10 kHz for 12 hours in a chamber filled with media containing <sup>35</sup>S-methionine. Unstimulated controls were incubated and labelled in an identical specimen chamber in the same incubator. Protein synthesis in electrically stimulated specimens relative to controls was assessed. Stress protein

synthesis was assessed by examination of gel fluorographs. Total protein synthesis was assessed by radiolabel incorporation.

No stress protein synthesis was induced for current densities up to  $30\text{mA}/\text{cm}^2$ , at frequencies of 1, 10, and 100 Hz and 1 and 10 kHz. This lack of stress response indicates that fields within these amplitudes and frequencies are not toxic to cartilage tissue, in the sense defined by the stress response. The maximum current density applied was at least an order of magnitude larger than fields applied clinically, and is many orders of magnitude larger than fields expected to be induced by environmental sources for frequencies less than 10 kHz. Therefore, these experiments do not suggest possible hazardous effects of electric fields which would be associated with synthesis of stress proteins.

An increase in total protein synthesis, as measured by  $^{35}\text{S}$ -methionine incorporation, was induced for high enough current densities ( $>10\text{ mA}/\text{cm}^2$ ). Electric fields, therefore, appear to act by some mechanism to change an important physiological function of the chondrocytes. The increase in incorporation depended on the position on the joint surface from which plugs were obtained, which resulted in a large variation in the response to electric fields within a single experiment. The mean increase in incorporation, however, increased with the magnitude of the current density.

The finding that relatively large current densities were required to measurably increase protein synthesis does not necessarily indicate that physiological current densities are unimportant. This model system displayed enough variation that small changes in synthesis (e.g.  $< 20\%$ ) were difficult to quantify. Such small changes can be important, particularly if the change occurs in a few specific proteins or over a long period of time.

The change in total protein synthesis indicates that electric fields may play a role in physiological regulation of chondrocyte metabolism. These results may have important implications regarding clinical applications of fields to alter growth and remodeling.

Thesis Supervisor: Alan J. Grodzinsky

Title: Professor of Electrical Engineering and Bioengineering

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## Chapter I

# Mechanisms of Interaction of Electric Fields on Biological Tissues

### 1.1 Introduction

Electric fields are generated naturally in biological tissues by mechanisms such as chemical diffusion and mechanical deformation. In some cases, the physiological role of such fields remains to be elucidated. Examples of such naturally occurring (endogenous) fields include: the currents associated with tissue injury or regeneration and currents generated by the mechanical deformation of bone and connective tissues.

In view of the close connection between endogenous fields and normal physiological function, it is reasonable to speculate that applied fields might alter tissue or cell function, whether in a beneficial or harmful way. Fields have been applied clinically, for example, to stimulate healing of bone and connective tissues [24,11]. Concern with the potential hazards associated with environmental field sources, such as power lines and household appliances (frequencies of dc and 60 Hz) and communications and radar systems (frequencies of approximately 10 kHz to 100 GHz) has led to studies on the effects of fields on biological tissues in these frequency regimes [32,159,98].

The motivation for investigating field effects in biological tissues, therefore, is twofold. The primary motivation is to understand the role of naturally occurring fields in tissue and cell function. A secondary motivation is to understand the effect of externally applied fields. These external fields can be fields applied intentionally in order to modulate naturally occurring processes (as in clinical applications) or can be fields in the environment.

The approach taken here was to develop a framework for understanding

electric field effects on physiological systems in terms of field amplitudes and frequencies:

(1) Field magnitudes and frequencies which might be of interest were identified. These are different for physiologically generated, clinically applied and environmental fields.

(2) Some possible mechanisms by which fields might interact with biological tissues were identified. The models were used to estimate field magnitudes and frequencies for which fields might produce important changes in some physiological parameter (e.g., ion concentrations) by each model mechanism.

(3) An experimental model was proposed to examine field effects on tissues over a range of amplitudes and frequencies. The model was field induced changes in stress protein and total protein synthesis in cartilage tissue.

The frequency range chosen for investigation ( $f < 10$  kHz) included the range of frequencies for physiologically generated fields (dc to a few kHz) and some of the range of frequencies of known externally induced fields (dc to 100 GHz) and was within the range (less than 100 kHz) for which the model mechanisms predicted field induced changes of 1-10% in some physiological parameters. Initial studies used current densities on the order of  $1 \text{ mA/cm}^2$ , since the model linear mechanisms suggested that current densities of this order would be required to produce 1-10% changes. The experimental model did not exclude the possibility of finding effects at lower current densities. In fact, however, current densities of greater than  $10 \text{ mA/cm}^2$  were found to be required to induce a change in protein synthesis in this experimental model.

Therefore, in this chapter, first naturally occurring (endogenous) and externally induced (exogenous) field magnitudes and frequencies are compared. Second, several specific interaction mechanisms are proposed and characterized in terms of the magnitudes and frequency of fields that might produce an important change in some physiological parameter. Finally, the experimental model is proposed, which is

field induced changes in stress and total protein synthesis in cartilage tissue. Stress protein and total protein synthesis is assessed by radioactive labelling of proteins, followed by gel electrophoresis (Chapter II, Methods). The model is then used to investigate field effects on tissues as described in the following chapters.

Mechanisms by which fields might act include thermal and non-thermal mechanisms. Thermal effects are those due to ohmic heating of the tissue and the resulting temperature elevations or gradients. Non-thermal effects are any others which cannot be attributed to heating. These could have a basis in electromechanical and physicochemical processes involved in interactions between transport, structural mechanics and chemical binding on the cell and membrane level, or in molecular physics and quantum mechanical processes involved in the configurations of the molecular components making up the tissue structure and the electrical properties of the tissue material.

## 1.2 A Comparison of Endogenous and Exogenous Field Magnitudes and Frequencies

Endogenous fields in tissues span a wide range of amplitudes and frequencies. Steady currents can be found in amphibian limb stumps after amputation and in mammalian tissues after injury or during regeneration. These injury or regeneration currents vary in magnitude from about  $10\mu\text{A}/\text{cm}^2$  in newt forelimb [19],  $5\mu\text{A}/\text{cm}^2$  near fractured mouse metatarsals [20] to  $300\mu\text{A}/\text{cm}^2$  near a cut in guinea pig skin [7]. It is thought that these currents are primarily of electrochemical origin, being associated with ionic gradients across cell membranes within epithelial tissues [19,7].

Electrochemical flows are also associated with the electrical activity of nerve and muscle tissues. Current densities as high as  $1\text{ mA}/\text{cm}^2$  are found near the surface of nerve and muscle fibers during propagation of action potentials [1]. Frequency components of action potentials along the nerve fiber can be as high as a few kHz.

Mechanical deformation of bone and soft connective tissues generates electric fields within these tissues. While the mechanism for such mechanical-to-electrical transduction is thought to be piezoelectric for the case of dry bone [59], it is now believed that electrokinetic (streaming) interactions give rise to the current densities measured in wet bone [167,78], and cartilage [76] under physiological conditions. Oscillatory streaming currents of  $1\text{-}50\mu\text{A}/\text{cm}^2$  were recently demonstrated in bovine knee articular cartilage subjected *in vitro* to physiologic loading amplitudes and frequencies (near 1 Hz) [53,55]. Based on theoretical models fit to the experimental data to date, streaming current densities as high as  $100\text{-}1000\mu\text{A}/\text{cm}^2$ , can be estimated to occur in the upper regions of the tissue when subjected to compression at higher frequencies within the physiological range for mechanical loading [56]. The range of frequencies for streaming currents is wide, from almost dc to higher than 1 kHz. (Since impact loading of cartilage may involve a wide range of frequencies, the accompanying streaming currents would presumably contain similar frequency



components).

In cartilage and bone, mechanical loading produces convective flow of interstitial fluid which entrains counterions through the highly charged extracellular matrix of the cartilage. Electrochemical gradients and even the cells themselves play a negligible role in the generation of these endogenous fields. It has been hypothesized, however, that such fields can react back on the cells to affect growth, remodeling and biosynthesis of connective tissues, [9,60] by coupling to cells directly or indirectly through the extracellular matrix.

The examples above show that physiologically generated currents are found in all four major tissue types: nerve, muscle, epithelial and connective. The range of endogenous current density magnitudes is quite extensive ( $1\text{-}1000\mu\text{A}/\text{cm}^2$ ). The range of characteristic frequencies is also large, and ranges between dc and approximately 1 kHz (Fig. 1.1).

Fields induced within body tissues by external sources, either clinical or environmental, span a similarly wide range of magnitudes and a wider range of frequencies compared to physiologically generated fields. Fields in the environment range from dc (e.g. dc power lines) to approximately 100 GHz (satellite and radar systems).

A useful reference for external environmental field magnitudes for frequencies above 3 kHz is the magnitude of internal field induced by electromagnetic radiation at power densities within the ANSI and ACGIH standards (Fig. 1.2). These are voluntary government standards for communications and industrial use. The maximum field magnitudes permitted at each frequency within these standards are based on levels of absorbed power, or equivalently, on ohmic heating within the body. Therefore, they represent a limit above which any field-induced effects which are non-thermal in origin will be secondary to effects related to thermal stress. However, local heating and thermal gradients at field exposures below these standards may still be pertinent to field effects.

# Typical amplitudes and frequencies of physiologically generated fields

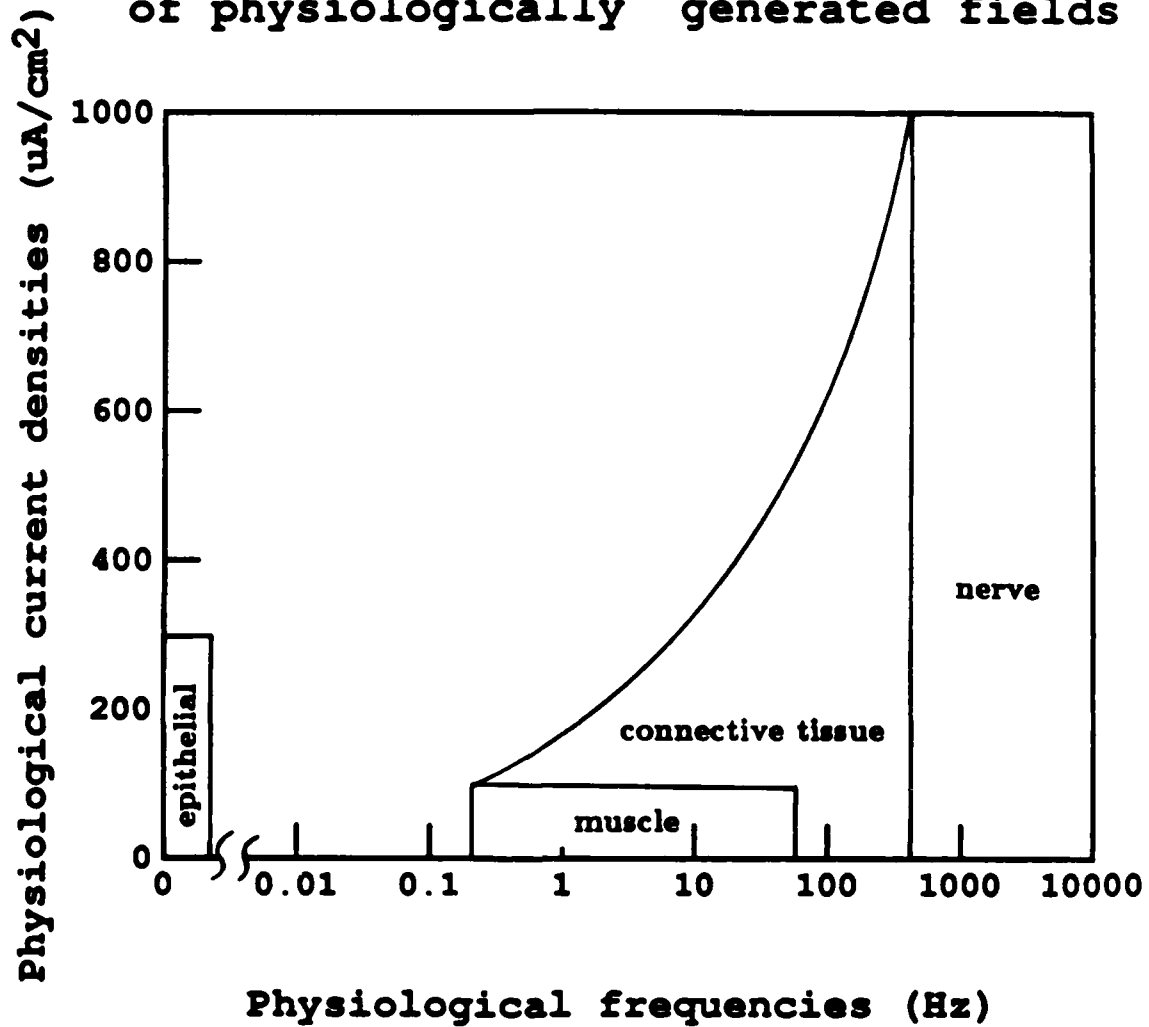


Figure 1.1: Endogenous field magnitudes and frequencies.

Lower frequency and dc field exposures are not yet regulated, but typical magnitudes of electric fields induced within tissues by external fields at these frequencies are smaller, as discussed following the calculation of internal field strengths. Some examples of field magnitudes and frequencies that are used clinically for cartilage and bone healing are given in Section 1.4.4.

The magnitude of the internal electric fields induced by incident power densities within the ANSI standards depends on the frequency. In the quasistatic limit, where the wavelength of the incident radiation is long relative to the largest body dimension, the body can be modeled as a conducting object in a uniform field. In the plane wave limit, where the wavelength is short relative to the radius of curvature of the body, internal fields can be estimated by geometric optics. In the intermediate frequency range, there is a resonance region (between 10-100 MHz), where the wavelength is approximately twice the body height. At the resonant frequency, whole body absorption of radiation reaches a maximum. Secondary resonances for absorption by body part occur near frequencies for which the wavelength is twice the characteristic dimension of the body part [61,62,186].

In the quasistatic regime ( $f \ll 1$  MHz), simple spherical and cylindrical models can be used to identify the salient features of the induced fields and estimate the induced field magnitudes (Appendix A.1). In this regime, the internal electric field is induced primarily by the external magnetic field, rather than the external electric field. The magnitude of induced field is proportional to frequency, and body radius. Constrictions in the body radius, as for example in the neck, cause an intensification of induced current density as demanded by continuity of current.

In the plane wave limit ( $f \gg 1$  GHz), currents are induced within a thin surface layer having a characteristic skin depth  $\delta = \sqrt{\frac{2}{\omega \mu_0 \sigma}}$  [101]. The thickness is on the order of 3 cm at 1 GHz and on the order of 3 mm at 100 GHz (based on tissue parameters given in [171]).

Near resonance (10-100 MHz), an estimate of local internal field magnitudes

can be obtained using the analysis of Hagman and Gandhi [61,62]. They developed a block model of man in order to include the actual geometry of the human body in a numerical calculation of absorbed energy, and tested the theoretical predictions with measurement of power absorbed in scale models. Their models show that in the 10-100 MHz range, the fields within the body are not at all uniform. For a plane wave incident perpendicular to the body axis, the parts of the body, in order of the maximum to the smallest magnitude of internal induced field were the legs, abdomen and neck, and the chest and head. The maximum internal fields induced by the maximum external fields within the ANSI standards between 10-100 MHz were calculated to be 60-90 V/m in the lower thigh, 30-50 V/m in the neck and lower abdomen, and approximately 20 V/m in the center of the head (at the maximum incident power density permitted by the ANSI standards of  $1 \text{ mW/cm}^2$  at resonance (70-80 MHz)).

Figure 1.2 summarizes the maximum incident power density (or field magnitude) permitted within the ANSI and ACGIH standards, and the corresponding magnitudes of the maximum internal fields induced by these external fields. The maximum induced fields will occur in regions of current constriction in the quasistatic limit, and at the surface in the plane wave limit. At intermediate frequencies, the location of maximum field strength depends on the dimensions of body parts relative to the incident wavelength.

The magnitudes of these induced fields for frequencies above 1 MHz are at least on the order of physiologically generated fields. The frequency range, however, is above the range associated with endogenous fields. For frequencies below a few kHz, internal electric fields induced by capacitive coupling decrease linearly with frequency (as shown with the simple quasistatic model). For the same amplitude of external field, therefore, currents induced will be on the order or lower than the endogenous currents. In clinical applications, however, induced field magnitudes might be as high as  $1 \text{ mA/cm}^2$ , for frequencies below a few kHz, depending on the

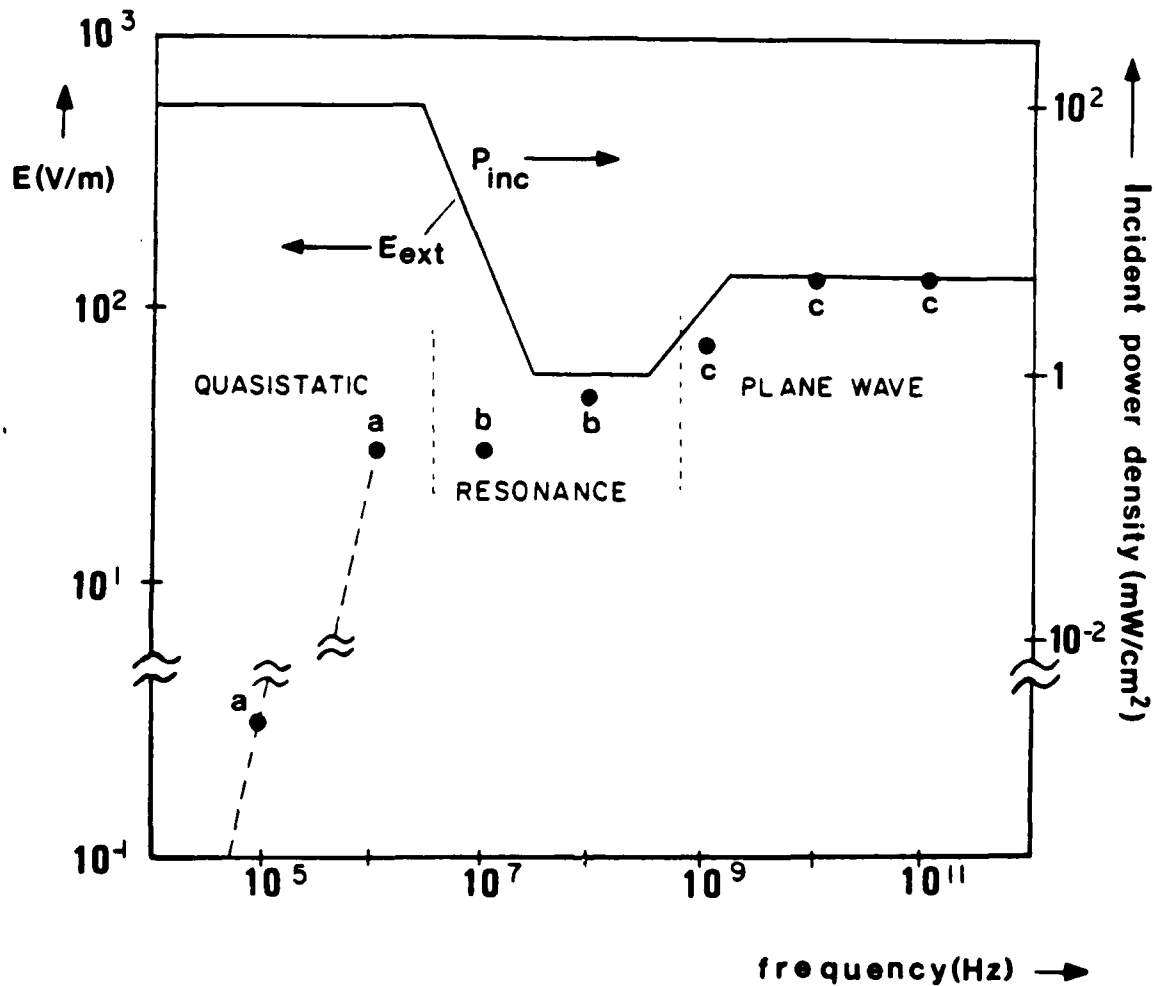


Figure 1.2: External and internal electric fields. The solid line is the peak field corresponding to the maximum incident power density permitted under ANSI ( $0.3\text{-}10^5$  MHz) or ACGIH (below 0.3 MHz) standards. The dashed line and points are the induced internal field strengths (a) within a spherical homogeneous conducting dielectric model of man using electroquasistatic methods ( $\sigma, \epsilon$  that of physiological saline), (b) computed at frequencies near resonance for whole body absorption [61,62], and (c) in surface regions of a human computed from a wave incident on the surface.

technique used to apply the field.

### **1.3 Characterization of Physical Mechanisms in Terms of Magnitude and Frequency**

#### **1.3.1 Introduction**

The magnitudes of internal tissue fields which are either physiologically generated or externally induced can be used to evaluate the magnitude of field effects on tissues by some possible model mechanisms. Electromechanical and physicochemical mechanisms by which electric fields might act include: modulation of transmembrane potential, membrane pore size and ionic and macromolecular concentrations in membranes and extracellular matrix; and field-controlled migration of macromolecules and cell membrane constituents. Since wavelengths corresponding to frequencies below a few GHz are larger than the dimensions of cells and macromolecules in the tissue matrix, quasistatic field analyses are appropriate for modeling these interaction mechanisms.

The magnitude of the physiological response to fields is expected to depend on the magnitude of the field in the tissue, and on the time scale associated with the frequency of the physiologically generated or applied field relative to the rate constants associated with the particular physical mechanism considered. Fig. 1.3 shows some time constants associated with electrical, electromechanical and physicochemical processes. The next subsections summarize theoretical models for field interaction that were used to motivate the experimental model. Based on these models, induced field magnitudes on the order of the endogenous fields, could lead to changes in transmembrane potential and ion concentrations in some frequency ranges that merit consideration. Field driven motion of charged molecules along the surface of a cell might also be important for sufficiently low frequency fields (less than 100 Hz).

## CHARACTERISTIC TIMES AND CORRESPONDING FREQUENCIES

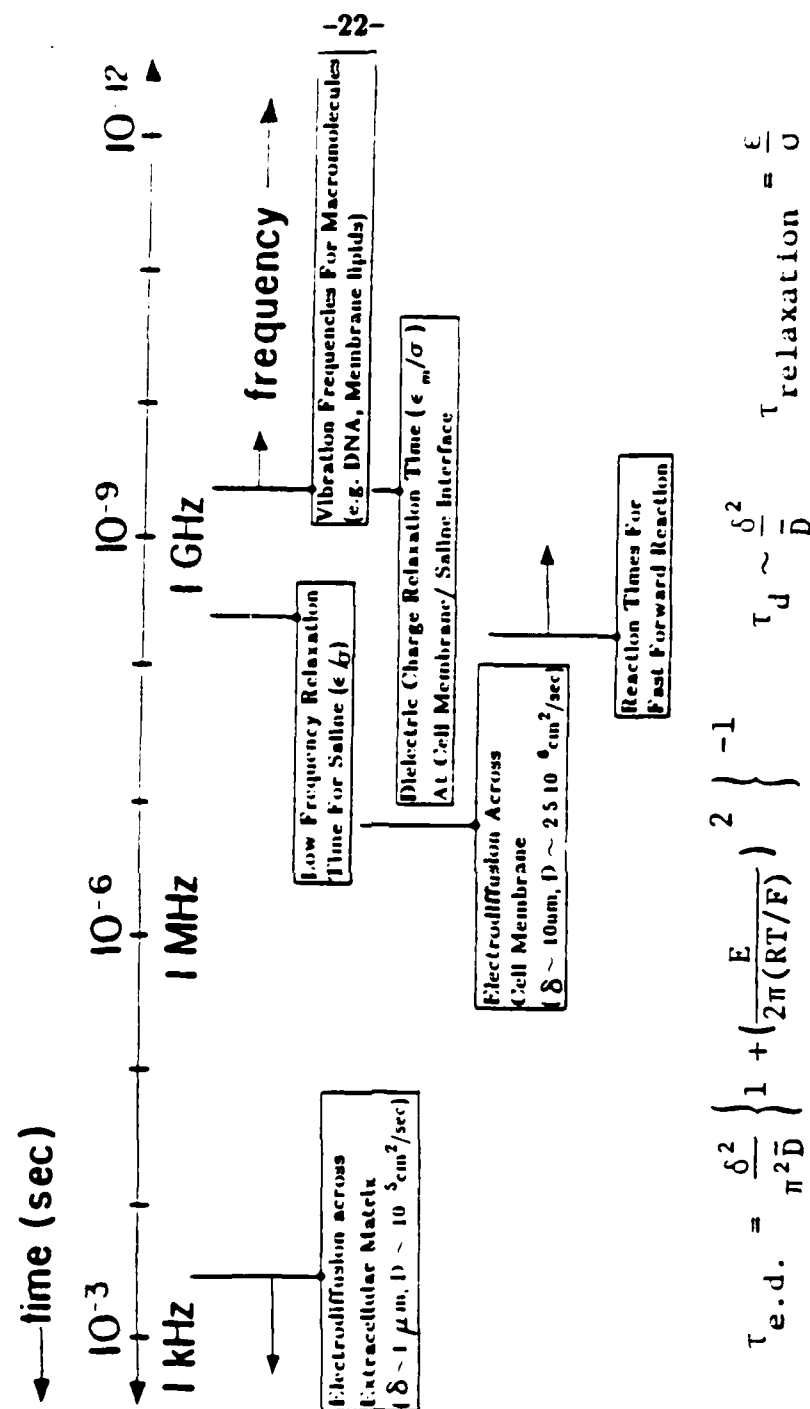


Figure 1.3: Time constants associated with electrical, electromechanical and physicochemical processes

### 1.3.2 Modulation of Transmembrane Potential

The induced change in transmembrane potential was calculated for a spherical shell model of a cell in a uniform applied electric field  $E_o$  (current density  $J_o$ ) (Fig. 1.4). The fields were calculated in the intracellular space ( $\sigma_i \approx 1$  mho/m,  $\epsilon_i \approx 80 \epsilon_o$ ), the cell membrane ( $\sigma_m \ll \sigma_i$ ,  $\epsilon_m \approx 1-10 \epsilon_o$ ), and extracellular space ( $\sigma_e \approx \sigma_i$ ,  $\epsilon_e \approx \epsilon_i$ ). Laplacian potentials were assumed for each region and appropriate boundary conditions on field and current were used (Appendix A.2). This problem has been analyzed extensively in the low frequency ( $f \ll 100$  kHz) or dc limit [169,157,187], where

$$\Delta \Phi_{mem} \approx -\frac{3}{2} E_o R \cos \theta \quad (1.1)$$

and in the very high frequency limit ( $f \gg 1$  GHz) where conductances are unimportant [189]:

$$\Delta \Phi_{mem} \approx \frac{\epsilon_{(i,e)}}{\epsilon_m} \delta E_o \quad (1.2)$$

where  $\delta$  is the membrane thickness and  $R$  is the cell radius. However, less attention has been paid to the intermediate frequency range, where relaxation times are in the range of the period of the applied field.

The induced change in transmembrane potential varies with position around the cell periphery and is the difference between the potential evaluated at the external and internal membrane surfaces at any angle  $\theta$ . The perturbation in transmembrane potential is larger at frequencies  $\omega \epsilon_e / \sigma_e \ll 1$  or  $f \ll 1$  GHz, for which the cell membrane behaves as an insulator, than at high frequencies. In the intermediate frequency range where the relaxation time,  $\epsilon_e / \sigma_e$ , satisfies the criteria:

$$\frac{R \omega \epsilon_m}{\delta \sigma_e} \approx 1 \text{ but } \frac{\omega \epsilon_e}{\sigma_e} \ll 1 \quad (1.3)$$

$$\Delta \Phi_{mem} \approx -\frac{3}{2} E_o R \left( \left[ 1 + j \frac{3 R \omega \epsilon_m}{2 \delta \sigma_e} \right] \left[ 1 + \frac{j \omega \epsilon_e}{\sigma_e} \right] \right)^{-1} \cos \theta \quad (1.4)$$



# MODULATION OF TRANSMEMBRANE POTENTIAL

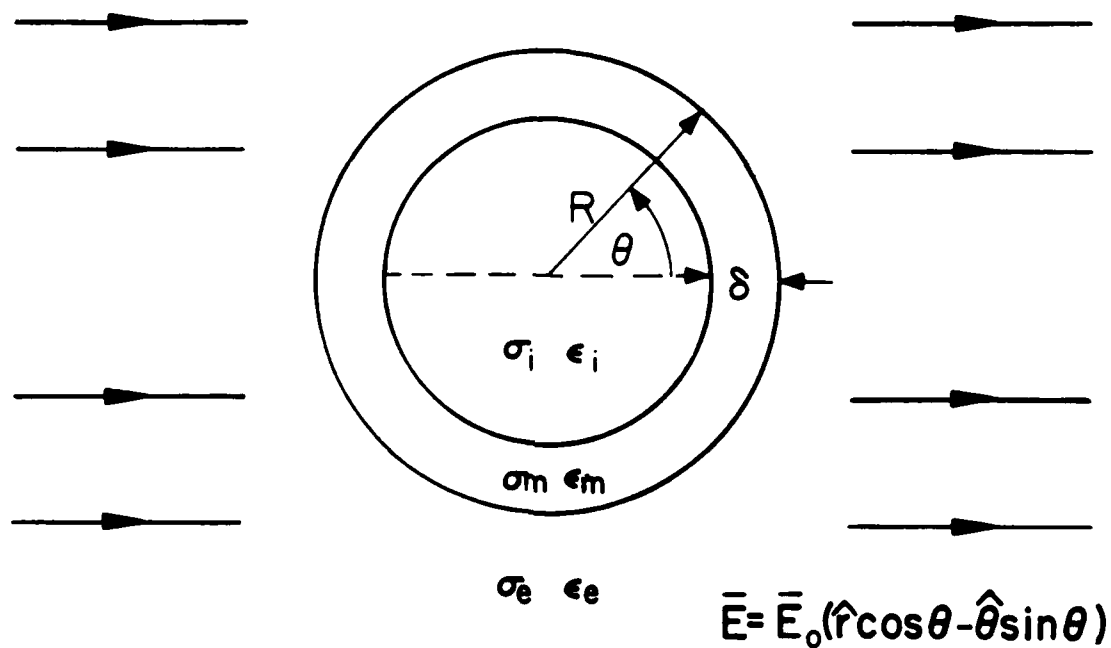


Figure 1.4: Spherical shell model for a cell used to find the change in transmembrane potential for an imposed field  $E_0$ .

and the corresponding change in membrane field is:

$$\Delta E_{mem} = -\frac{\Delta \Phi_{mem}}{\delta} \quad (1.5)$$

For a uniform field  $E_0$  corresponding to endogenous or applied current density magnitudes of  $1 \text{ mA/cm}^2$  (an electric field of approximately  $0.1\text{-}0.2 \text{ V/cm}$ ), the change in membrane potential for a spherical cell  $10 \text{ }\mu\text{m}$  in radius can be 1% of the initial transmembrane voltage (approximately  $V_T$ ), for frequencies below  $10 \text{ MHz}$ . (Chondrocytes, the cells of cartilage are essentially spherical with radii on the order of  $10\text{ }\mu\text{m}$ .) Current densities on the order of  $1 \text{ mA/cm}^2$  can be induced, as shown earlier, by external fields within the ANSI standards at about  $1 \text{ MHz}$ . Since  $\Delta \Phi$  induced is proportional to the cell size ( $R$ ), larger changes in transmembrane potential would be induced for larger cells. In addition, shape factors and orientation for non-spherical cells could result in a further increase in this change, even when comparing cells of the same maximum dimension.

Cooper [36] has suggested that the potential induced by dc fields, at the ends of elongated cells or cells electrically linked by gap junctions may be an order of magnitude larger than the potential induced across the cell membrane of a shorter cell with the same diameter (assuming typical values for the resistance in extracellular and intracellular fluid, and for the membrane). His analysis is valid as long as the cell membrane behaves as an insulator, so that similar shape dependent increases in the field induced change in transmembrane potential would be expected for ac fields in the low frequency and intermediate frequency regimes.

There is some experimental evidence *in vitro*, indicating that cells respond to changes in membrane potential of a few percent [157,158]. Nerve cells have been polarized by external fields so that they orient or grow along the axis of the imposed field [158,35,96]. Fucus eggs have also been found to orient along the axis for low frequency pulsed fields (pulse widths of  $10\text{-}1000 \text{ msec}$ ) as well as for dc fields [157]. In the pulsed fields, they orient along the axis, with an approximately

equal number pointing toward each electrode, while in the dc field, the orientation is unidirectional. Bidirectional or unidirectional orientations of about 50% of the cells were obtained for pulsed and dc fields respectively for fields near 4 V/cm. Miller [146] has claimed that a change in transmembrane potential of 5-10 mV (i.e., a 10% change) may trigger a cellular response to 60 Hz fields. A change in transmembrane potential might also have an effect on neuron firing of nerve cells. Some investigators have reported that radiofrequency radiation affects neuron firing rates and transmitter release for a range of incident power densities, and several different frequencies [32].

### 1.3.3 Modulation of Membrane Concentration by Electrodifusion

Concentration gradients of ionic species exist both within the extracellular matrices of biological tissues and across cell membranes. Characteristic dimensions over which concentration gradients are known to exist in human tissues and cells, include those associated with cell membranes (5-10 nm), basement membranes ( $\delta \approx 20$ -50 nm), whole cells ( $\delta \approx 10 \mu\text{m}$ ), and an extensive extracellular matrix, such as corneal stroma ( $\delta \approx 0.5 \text{ mm}$ ) and articular cartilage ( $\delta \approx 1 \text{ mm}$ ). Electric fields and their associated current densities within biological tissues might change these nonuniform ionic concentration profiles by the mechanism of electrodifusion. That is, an imposed field might alter the previously existing balance between the flux of ions due to diffusion and the reverse flux of ions due to migration driven by the field associated with charge separation.

The theory of electrodifusion [34] was used to investigate changes in ionic concentrations and fluxes within and across membranes and tissues in response to local electric currents (Appendix A.3). The relative change in concentration of ionic species in the central region of a charged matrix or membrane (supporting a concentration gradient) in response to a step change in local electric field was calculated to be on the order of  $(E\delta)/(RT/F)$ , for large initial gradients (greater

than 10:1), where  $F$  is the Faraday constant,  $R$  is the gas constant, and  $\delta$  is the thickness of the tissue matrix or membrane. An example is shown in Fig. 1.5. Significant modulation of gradients across very small lengths, such as a cell membrane thickness, will therefore require very large local field magnitudes ( $2.5 \cdot 10^5$  V/m for  $E\delta = 0.1V_T$ ). Conversely, the same 10% change in concentration profiles across larger length scales (about 1 mm) in connective tissue matrices, for example, will require lower field magnitudes ( $E \approx 1$  V/m). Thus, in terms of the magnitude of effect produced for a given internal tissue field magnitude, larger lengths are favored.

The kinetics associated with the establishment of the new concentration profile, however, impose another restriction on the length scale. The new concentration profile is established in a time characterized by the electrodiffusion time. The electrodiffusion time constant describing the temporal evolution of changes in concentration profiles in response to a change in electric field is,

$$\tau \approx \frac{\delta^2}{\pi^2 D} \left[ 1 + \left( \frac{E\delta}{2\pi RT/F} \right)^2 \right]^{-1} \quad (1.6)$$

The electrodiffusion time is nearly independent of the field for the small field magnitudes ( $E\delta \ll RT/F$ ) considered here. For typical ion diffusivities, electrodiffusion times will be shorter than the period at 100 MHz (1 Hz) only for lengths smaller than 5 nm ( $50\mu\text{m}$ ). Therefore, the magnitude of change in concentration induced by a given field magnitude is favored by large distances (on the order of dimensions of the extracellular matrix of connective tissues), and the response time favors smaller distances (on the order of the cell membrane thickness). For a given frequency and field magnitude, the length scale over which the maximum change will occur is approximately such that the diffusion time is on the order of the time period of the field ( $\delta^2 \approx \pi^2 D/f$ ). In the case of a cell membrane or extracellular matrix, rectification of the small change produced during one period of the field can produce

# ELECTRODIFFUSION INDUCED MODULATION OF IONIC CONCENTRATION

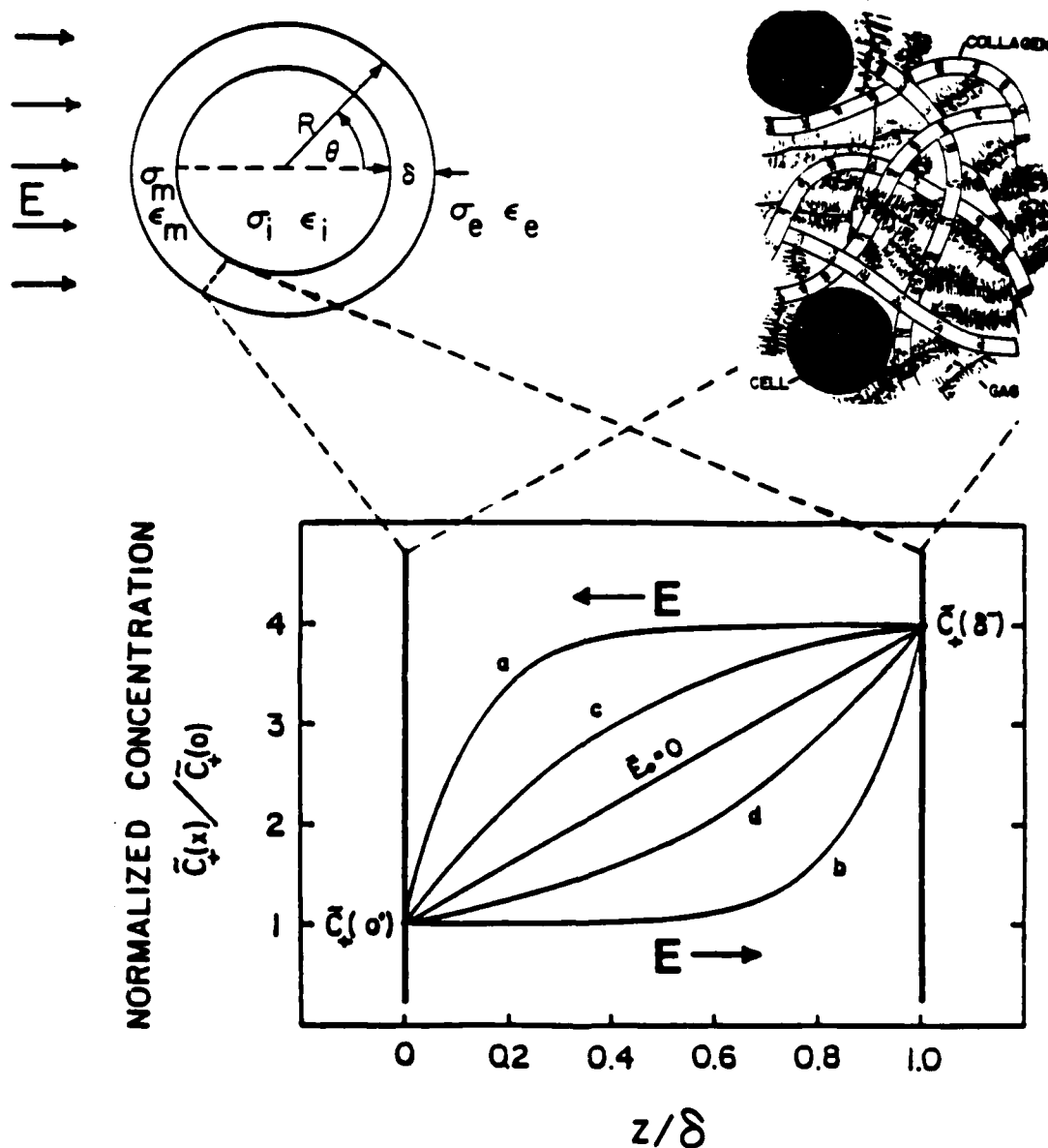


Figure 1.5: Electrodifffusion induced change in co-ion concentration within a positively charged membrane or tissue matrix of thickness  $\delta$ , for a concentration gradient of 4:1. Ratios of  $|(E\delta)/(RT/F)|$  are (a,b) 8; (c,d) 2: field direction as shown.

a larger change in concentration for fields applied over a long enough time.

#### 1.3.4 Rectification of Transmembrane Flux

Both the induced change in transmembrane potential and first order change in ion concentration are linear and reversible processes; that is, any effect produced in the first half cycle is reversed in the second half. Thus rectification is a major recurrent issue if ac electromagnetic fields are to effect changes in cells or biological tissues. Rectification is used here to mean production of a time average value of some physical parameter by an alternating (zero-time average) drive. It may be caused by special properties of the biological medium (e.g. uni-directional hindered transport) or by natural or field-induced asymmetries (e.g. concentration gradients).

Rectification of time-varying transmembrane fluxes was modeled for an imposed time-varying, spatially uniform electric field across a membrane (Appendix A.4). The ion current is a nonlinear function of the potential due to electrodiffusion induced changes in intramembrane concentration profiles, so that over one period the current has a non-zero time average. This is an example of the rectification due to an existing asymmetry (the concentration gradient). (Rectification of potential in excitable cells comprises a specialized body of literature, and is modelled using more complex coupling between transport of ionic species. Mechanisms for rectification in excitable membranes will not be discussed here.)

The non-zero time average change in single ion transmembrane current was calculated for an imposed spatially uniform field having a square wave time dependence. In addition to this imposed time-varying field, the physiological resting potential is consistent with an initial membrane ion concentration profile. The imposed field induces a change in concentration profile, with a concomitant change in current density,  $J = \sigma(E') E'$ , where  $E'$  is the imposed field. The induced current density was assumed to be divergence free (i.e. the ion concentration varies in quasi-equilibrium with the field), valid for incident fields at frequencies whose

period is much longer than typical diffusion times across the membrane (i.e.  $f \ll 10$  MHz for a cell membrane).  $J$  was determined as a function of the concentrations at the membrane boundaries and the imposed field, and was found to be a nonlinear function of the imposed field. An expansion of  $J$  in terms of the imposed field in the small field limit, showed terms that have a non-zero time average.

For typical  $K^+$  concentrations and an imposed time varying transmembrane potential that is 5-10% of the resting potential, the magnitude of the time average ion flux density was calculated to be a few percent of the magnitude of the ac flux. Thus, external fields in the frequency range below 10 MHz might be capable of producing a change in intracellular ion concentrations over a long enough time.

Changes in the concentration of physiologically active ions (e.g. calcium) may not need to be large to affect physiological function. A model for the mineralization of bone [64] suggests that very small changes in calcium concentration can result in very large changes in mineralization and the process of nucleation. Kaczmarek [104] suggested that calcium binding and release in cerebral tissue is very sensitive to changes of less than 40% in free calcium concentration.

Experimental work by Pickard's group [163,8,151] demonstrated rectification of potential across a cell membrane for applied ac fields less than 10 MHz. They measured a dc offset in the cellular transmembrane potential of *Chara* and *Nitella* induced by an ac external field at frequencies below 10 MHz. Above 10 kHz, the offset was measured to be approximately inversely proportional to frequency, and proportional to the applied voltage squared. Below 6 kHz, the offset was weakly dependent on frequency, and approximately proportional to the applied voltage squared. The frequency dependence, and amplitude dependence of the offset, therefore, are similar to the square of transmembrane potential calculated to be induced across a cell membrane by an applied ac field (in Section 1.3.2).

### 1.3.5 Modulation of Pore Size

Endogenous or induced fields might affect the transport of ions, nutrients and biosynthetic products through specialized cell membrane channels, by changing the morphology, hydration, and equivalent pore size of these channels. In the extracellular space, similar field induced changes in matrix permeability could affect the transport of ions and nutrients between cells and capillary beds and across connective tissue matrices.

A simple one dimensional model (Appendix A.5) was used to examine the possibility that fields induced in the neighborhood of cells might affect transport by modulation of the equivalent pore size of the membrane (cellular or extracellular). Because of the phenomenon of restricted diffusion (the hydrodynamic interaction between solute and pore wall for solutes with radii of about 10% of the pore radius or larger), very small changes in pore radius can cause large changes in transport of solutes [14].

The model used to estimate the effects of applied fields on the size of equivalent pores in a cell membrane or extracellular matrix represents the walls of the pore by two charged parallel plates, each of which supports an electrical double layer. A change in double layer repulsion force between the constituents of the pore walls (i.e., the parallel plate molecules), due to an imposed field could lead to a change in the membrane pore size. The pore size is approximated therefore, by the interplate spacing  $W$ . Pore size is on the order of a Debye length ( $1/\kappa$ ) for pores within the cell membrane, so that  $\kappa W$  is of order one. In the extracellular matrix, intermolecular spacing is typically on the order of, or larger, than a Debye length ( $\kappa W \geq 1$ ). This model predicts that for  $\kappa W \geq 1$  the current densities calculated to change pore spacing by 10% are several orders of magnitude greater than  $1 \text{ mA/cm}^2$ , even for small surface potential  $\phi$  ( $\kappa\phi \approx 10^6 \text{ V/m}$ ). Thus, this model suggests that pore size is not modulated by either endogenous fields within the tissue space or by fields induced by external fields within the ANSI limits. (The large endogenous fields



which exist across cell membranes are known to affect transport.)

The above model is linear, and therefore is consistent with a sinusoidally varying pore size. However, since solute radius may be on the order of pore radius for many important solutes, the phenomenon of restricted diffusion can introduce a strong nonlinearity in the transport of solutes through a periodically varying pore structure. Diffusion times across a 5 nm membrane are  $10^{-8}$  to  $10^{-9}$  seconds. Transport might therefore be affected even by a sinusoidal change in pore size, as long as the pore can mechanically respond to the electrical force. Inertia at higher frequencies will eventually limit the pore's mechanical response. Based on the magnitude of internal field values required, however, changes in pore size would appear to be very small.

### 1.3.6 Electrophoresis

Jaffe [102] first suggested that electrophoresis on the surface of cell membranes could be a possible mechanism underlying the effects of electric fields in developing tissues. It is now accepted that cell membrane constituents may exist in a fluid matrix, with constituents capable of rapid translational and rotational movement [169]. Such constituents include cell surface receptors, proteins which bind specifically only to certain molecules (ligands) in the extracellular space. Cell surface receptor movement is an important part of the transduction mechanism which couples ligand-receptor binding to cellular responses [41]. Insulin-induced receptor clustering, leading to increased glucose utilization by fibroblasts [116] is an example of this phenomenon. Poo et al. observed migration of receptor proteins for dc fields on the order of 1-10 V/cm [169]. McLaughlin and Poo [139] observed that a dc field of 10 V/cm applied for 30 minutes could induce receptors to aggregate on the surface of *Xenopus* myocytes (toad muscle cells).

Electrophoresis of charged macromolecules might affect motion within the extracellular space, or within the cell. Cooper has demonstrated that applied dc

fields of 0.2-0.4 V/cm within crayfish neurons can drive electrophoretic motion of fluorescent charged dyes within the axon [37]. AC fields might similarly be capable of eliciting electrophoretic motion, either within the membrane, or within the cell cytoplasm that might have biological consequences. The field in the cytoplasm of a long cell or in cells coupled by gap junctions is nearly equal to the imposed field [189,36]. Thus the current densities flowing in the axoplasm of a long cell, such as a nerve cell could be large on the physiological scale. For a field of 100 V/m (1 V/cm), the current density in the axoplasm, given by  $J = \sigma E$ , is 10 mA/cm<sup>2</sup>.

The velocity,  $U$ , of ions and biological particles driven by an imposed field can be represented by the constitutive relation:

$$U = bE \quad (1.7)$$

where the mobility,  $b$ , of the particle can be measured. Typical ionic mobilities are on the order of  $5 \cdot 10^{-3}$  to  $5 \cdot 10^{-4}$  cm<sup>2</sup>/V-sec in saline. The mobilities of larger charged particles, such as proteins, are typically an order of magnitude smaller. For particles that can be modeled as isolated spherical bodies, such as cells, or globular proteins, electrophoretic motion can be modelled using stress balance and charge conservation over a unit surface area in the electrical double layer [74,119].

Induced bulk electrophoresis of biological cells or macromolecules can be evaluated by comparing the drift velocity to an initial velocity, such as the diffusion velocity (pertinent to diffusion of neurotransmitters across the synaptic cleft for example) or by comparing the distance travelled in one half period of the field relative to some length of interest. The ratio of drift to diffusion velocities is

$$\frac{U_{drift}}{U_{diff}} = \frac{E\delta}{2RT/|z|F} \quad (1.8)$$

based on the Einstein relation [74], so that  $E\delta$  must compete with the thermal voltage, and the perturbation will be small (less than 10% for distances  $\delta$  less than

5 $\mu$ m) for internal fields on the order of 1 V/cm, when  $|z| = 1$ .

The distance travelled in one half period is  $b \langle E \rangle T/2$ , where  $\langle E \rangle$  is the time average field in one half cycle. This distance is largest for low frequency fields. For the case of receptors or particles moving within a membrane, small distances (1nm) may be important. The mobility for Con A receptors [169] was measured to be about  $1.9 \cdot 10^{-11}$  m<sup>2</sup>/V-sec. Thus for fields of 1-10 V/cm, such receptors might be expected to move distances larger than 1 nm within one half cycle, for frequencies below 10 Hz. Although Poo's studies were done exclusively in dc fields, the formation of receptor aggregates [168], which suppress back diffusion when the field is turned off, suggests a possible field rectification mechanism for very low frequency ac fields.

### 1.3.7 Summary of Models in Terms of Amplitude and Frequency

The electromechanical models discussed above indicate that in terms of magnitude of internal field required to induce a possibly important physiological change, fields magnitudes on the order of 1mA/cm<sup>2</sup> (0.1 V/cm) are required. In terms of frequency, such mechanisms might be important for fields whose frequency is below 10 MHz, over frequency ranges determined by the characteristic times of each mechanism. Specifically, at frequencies of less than 10 MHz changes in transmembrane potential might be important, as determined by the charge relaxation times at the cell surface. At frequencies of less than 10 MHz changes in concentration profiles might be important across dimensions on the order of a cell membrane, or at frequencies of less than 1 kHz, across dimensions on the order of an extracellular matrix (1  $\mu$ m), as determined by the electrodiffusion time. At frequencies of less than 100 Hz, electrophoresis along the cell surface might be important, as determined by the distance travelled by a typical molecule during one period of the field.

In the next section, an experimental model is proposed to test the hypothesis

that electric fields within these magnitudes and frequency regimes can interact with certain cells and tissues to alter their biological behavior.

#### **1.4 Experimental Model**

##### **1.4.1 Model to Examine Dose Response in Terms of Magnitude and Frequency**

The experimental model chosen to examine field interaction with biological tissues was field induced changes in stress protein synthesis and total protein synthesis in cartilage tissue. Disks of articular cartilage were maintained in organ culture for 5 days and then placed in an electrical stimulation chamber and either exposed to current densities (experimental specimens) or to no current (control specimens). Protein synthesis, specifically stress and total protein synthesis, in the exposed specimens during electrical stimulation was compared to synthesis in the control specimens. Stress proteins serve as a marker for a field induced toxic stress on the cells, and in addition they serve as a control for thermal effects. Total protein synthesis serves as an additional marker for field induced changes in cellular metabolism.

The theoretical models motivated in part the choice of cartilage as the tissue model. Cartilage is representative of an important class of tissues—the connective tissues—found throughout the body. Physiologically generated fields are normally present in cartilage [55,56], and ion movements in the tissue are important to normal articular function. Thus, the type of mechanism developed in the theoretical discussion is part of the physiological function of cartilage. Further, the physiologically generated electric fields found in cartilage loading has motivated previous work with cartilage, so that the tissue has been characterized in terms of mechanical properties, total protein and GAG synthesis in culture, and variations in the properties of the tissue matrix across the joint surface. One of its characteristic properties, its high extracellular water and ionic content, facilitates calculation of applied field

within the tissue in culture media, since the electrical conductivity of cartilage is nearly the same as physiological saline. Finally, one of the markers chosen to assess the effect of electric fields on cartilage cells, stress protein synthesis, had been observed previously in heated chondrocyte cell cultures, and in chondrocytes isolated from osteoarthritic cartilage.

The theoretical models for possible mechanisms of interaction of electric fields on biological tissues also motivated the choice of electrical stimulation parameters. First, the frequency regime for experimental investigation was chosen based on the characteristic times associated with the mechanisms discussed. The frequencies corresponding to the characteristic times below which these mechanisms might be important were below 10 MHz. Thus, the experimental frequency regime was chosen to be between 1 Hz and 10 kHz. Second, the initial field amplitudes were chosen to be approximately 1 mA/cm<sup>2</sup>, based on the estimates of field magnitudes required to produce a change by these model mechanisms.

The theoretical models helped to motivate the choice of probe, stress protein and total protein synthesis, to be used to gauge the effect of electric fields on cartilage. The models had shown that over such a wide range of frequencies, several transduction mechanisms might be important, so that an experimental probe should not be mechanism specific. It should be sensitive to small perturbations, and responsive to dose (e.g., the magnitude of the induced effect). Stress protein synthesis fulfills these criteria to some degree. Stress proteins are synthesized in response to a variety of toxic agents, (heat, anoxia, heavy metal ions) [6,5,94] and so their synthesis is not mechanism specific. The stress response is sensitive, in that cells synthesize stress proteins in response to a few degree change in temperature [91,132] and small ( $\mu$ M to mM) changes in external concentrations of toxic agents [94]. The response is dose dependent. The rate of stress protein synthesis depends on the intensity of the stress, (e.g., the elevation of the temperature rise or the concentration of the toxic agents) and its duration. Furthermore, stress protein

synthesis is easily observed and quantitated by labelling with  $^{35}\text{S}$ -methionine, followed by gel electrophoresis and autoradiography. For these reasons, synthesis of stress proteins in response to applied electric fields was chosen as an experimental marker to identify field magnitudes and frequencies which might stress cells.

Total intracellular and extracellular protein synthesis was used as an additional probe of possible electric field effect, in particular, as a probe for stimulatory, rather than toxic effect. Stress response is primarily a marker for toxic effects. However, some research linking stress protein synthesis with stages in cell growth or tissue development (see next section), as well as work showing that some heat induced proteins are involved in normal protein degradation, suggests that stress proteins may have a larger physiological role, and may represent a response to alterations of cell state in addition to response to toxic agents.

The remainder of this chapter reviews some of the literature on the experimental markers and experimental tissue model. First the stress response is reviewed—with an emphasis on its use as a *marker for field effect*. Some of the literature on the experimental tissue model—cartilage—is then also reviewed, with an emphasis on normal protein synthesis in cartilage tissue, as background for field induced changes in protein synthesis. Finally, previous work investigating the effect of applied electric fields in cartilage and chondrocyte cell culture is reviewed with an emphasis on experimental design.

### 1.4.2 Stress Response

#### Overview

The stress response, also called the heat shock response, since it was first observed in response to heat, is the response mounted by cells to a variety of toxic agents. It is characterized by the synthesis of a particular set of proteins, the stress proteins, and under some conditions, by a decrease in normal protein synthesis. The stress proteins are highly conserved in evolution from bacteria to higher eukaryotes (e.g.,

animal and plant cells) [6,40,51], and are thought to play a role in protecting the cells from the diverse effects of stress. The mechanism of protection is thought to be reformation and degradation of the agent-induced abnormal proteins [51].

Many studies of stress response have been done using either *Drosophila* (fruit fly) cells or tissue cultures, the bacteria, *E. Coli*, or yeast, since these cells are easily maintained and manipulated. Yeast cultures have the further advantage that they can be genetically manipulated. Mammalian and avian cells, and to a lesser extent mammalian and avian tissues have also been used. This section briefly describes the stress response characteristics that are most pertinent to its use as a probe for the effect of electric fields on tissue, with an emphasis on the response in mammalian tissue or cell cultures and avian (chick embryo) cultures, where heat shock proteins have been shown in a number of studies to be induced under the same conditions as mammalian cells [103,107,94,93,122]. The characteristics which are most pertinent to its use as a probe include:

- (1) the dose response and kinetics of stress protein synthesis in response to the various toxic agents, as an indication of the sensitivity of stress response as a probe;
- (2) indications of the type of mechanism which might induce a stress response; and
- (3) the stress response observed in chondrocyte cell culture, as reported by previous investigators.

The kinetics of induction, are pertinent to the choice of electrical stimulation protocol, especially, the duration of the stimulation. Specifically, these include the exposure time required to induce stress proteins, and the persistence of stress protein synthesis both during and post exposure to the toxic agent.

Indications of the type of mechanism which could induce a stress response include how the inducing agents act on cells or cell constituents, and the function of stress protein synthesis.

The pattern of stress response depends to a large extent on the specific stimulus applied, the inducing agent, the dose, and the length of exposure. It should, therefore, be possible to compare field induced synthesis to synthesis by other agents (especially heat). In the event of field induced stress protein synthesis, the dose response and kinetics of field induced synthesis might be compared to the dose response and kinetics of other agents in the same tissue under the same culture conditions. Therefore, the dose response and kinetics of stress protein induction in cartilage by another agent, such as heat, have to be examined.

### Dose Response and Kinetics

Many toxic agents induce stress protein synthesis (reviewed by Ashburner [5] for *Drosophila*, and by Hightower [94]). These include: heat (either *in vitro* or *in vivo* [94,6,25,86]); LSD (*in vivo*), which can lead to hyperthermia [25]; amino acid analogs such as L-azetidine-2-carboxylic acid (AzC) or canavanine [196,107,94]; transition metal ions (such as arsenite, copper, or cadmium) [103], which act on sulfhydryl containing targets; metal binding drugs, (such as kethoxal bis(thiosemicarbazone) (KTS) and disulfiram) [121,94]; anoxia (including ligation of large vessels *in vivo*) [42]; viral infection [26,38,94,108]; tissue trauma [42,94], (by means of resulting stressors which might include: a change in O<sub>2</sub>, ischemia— or vascular injury [209]), mechanical damage, release from growth inhibition, or chemical mediators of inflammation); and aortic banding (*in vivo*) [86] (possibly by means of stresses associated with acceleration in cellular metabolism or cellular acidosis). In general, these agents are lethal in high enough doses.

The stress proteins are highly conserved between species [87,40], but the kinetics of induction vary considerably between species [40,107]. Within a single species, the 70 kD stress protein has been shown to have small differences (in peptide mapping, or in charge variants) depending on the tissue type (e.g. in rat [94,212]), and on the inducing agent (e.g. canavanine or tissue excision [42,94]).



The different stress proteins, typically referred to by molecular weight (e.g. SP70), have different levels of induction and different kinetics in response to a given toxic agent. The kinetics vary in time to induction of stress proteins, time to maximum induction, and persistence of induction (during or after removal of the toxic agent). The induction of any one stress protein depends on the agent and on the dose (e.g. concentration or duration of exposure). Thus, although the number of stress proteins induced in any one species is limited, the pattern of stress protein induction (in terms of the specific stress proteins induced and the kinetics of stress protein induction) can vary considerably [5,40,124,185]. However, for a given tissue under defined conditions, the response to the same stimulus is reproducible.

**(a) The different stress proteins are not expressed coordinately**

Differences exist in the dose which gives rise to the maximum induction of the individual stress proteins. For example, different  $\mu\text{M}$  concentrations of sodium arsenite, were required to induce the maximum level of different stress proteins in chick fibroblasts, [103] for a fixed incubation time.

The time to induction of a particular stress protein can range from a few minutes to several hours. In chick embryo cells exposed to disulfiram ( $0.012\mu\text{M}$ ), for example, three of 4 stress proteins were induced within 2 hours, while the fourth 35 kD, was not induced until greater than a 6 hour exposure [121]. Similarly, Levinson [122] found that for a 3 hour exposure different stress proteins were induced depending on concentration of kethoxal bis(thiosemicarbazone) in chick embryo cell culture. For a single concentration ( $2.8 \cdot 10^{-7}\text{M}$ ), the 70 kD protein was induced first. Then by 12 hours, synthesis of the 70 and 20 kD proteins was equal, and another kD protein appeared between 10-12 hours.

The time to reach the maximum rate of synthesis the time for a subsequent decrease in rate of induction also varies over a wide range for the different stress proteins, from a few hours, to more than 12 hours. In chick fibroblasts exposed to sodium arsenite ( $50\mu\text{M}$ ), all stress proteins appeared within 1 hour. While after 24

and 48 hours the 27 kD stress protein was still prominently synthesized, synthesis of both normal and other stress proteins was depressed [103]. When exposed to canavanine, synthesis of two stress proteins reached a plateau in 2.5-3hr, while synthesis of the third protein did not plateau until 5-6 hours [93] .

**(b) The pattern of stress response depends on the stimulus**

The inducing agent and dose govern the maximum level of induction for different stress proteins [208] and the kinetics. Both have been used to compare the response to different inducing agents. Welch compared the stress response induced by exposure to AzC for 8 hours to the stress response induced by incubation at 43°C in HeLa and gerbil fibroma cell cultures [208]. He found differences both in the amount of the 70 kD protein induced relative to other stress proteins and in depression of normal synthesis. Johnston [103] found that in chick fibroblasts and human foreskin culture, the kinetics for stress protein induction were different for cadmium and copper, and that the kinetics for induction by arsenite were similar to induction by cadmium.

The time to induction varies from a few minutes to a few hours, depending on the stimulus. Induction by heating takes less than a few minutes (chinese hamster fibroblasts [125] or HeLa cells [28]). Induction by tissue excision begins 30 min after tissue excision (in rat heart, brain, kidney etc. [94,42]). Induction by incubation in an amino-acid analog (HeLa cells exposed to AzC) [196] requires several hours.

With continuous exposure to the inducing agent, the time to reach maximum synthesis of stress proteins, varies from a few hours (exposure to heat, [91]) to over 12 hours (exposure of chick embryo to canavanine, [107]), depending on the inducing agent and on the dose. Under some conditions, stress protein synthesis declines after the maximum, while under other conditions the stress protein synthesis reaches a steady state.

Hickey et al. [91] found that stress protein synthesis in HeLa cells exposed to 42°C reached a maximum 3-5 hour into treatment, and then decreased to a low

level by 8 hours. Thomas [196], found that stress synthesis in HeLa cells continued to be strongly induced by AzC for up to 16 hours of exposure.

Schlesinger reported differences in kinetics depending on the elevation of the incubation temperature [185]. Synthesis of the SP70 stress protein in chick embryo cells exposed to 43°C ceased after 2 hours, while in cells exposed to 45°C it did not.

The time to recovery (return to normal synthesis) after removal of the stimulus is also stress protein, inducing agent and dose specific. The recovery time ranges between a few hours (e.g., in chick embryo cells following a 3 hour canavanine treatment [94]) to greater than 12 hours (e.g., in chick embryo cell following a 2 or 4 hour kethoxal bis(thiosemicarbazone) treatment [122]).

#### **(c) Stress Protein Synthesis is Accompanied by Changes in Normal Protein Synthesis**

Some inhibition of total synthesis and synthesis of normal proteins, often accompanies induction of heat shock proteins. Similarly to induction of the stress proteins, the degree to which total synthesis and normal synthesis are affected, and the kinetics of inhibition depends on the species [107], on the inducing agent [107,42,91,208], and on the dose [91,124,122]. Recovery of total protein synthesis can precede the decline of stress protein synthesis [93]

Currie [42] found that in the rat, *in vivo*, when stress protein synthesis was induced by heat (42°C for 15 min) total protein synthesis was depressed. When stress proteins were induced by ligation of common carotid arteries, overall synthesis was not depressed. Welch [208] compared normal synthesis in HeLa cells exposed to AzC (5 mM) to cells incubated at 43°. After 12 hours there was little synthesis of normal proteins in cells exposed to AzC; almost all synthesis was stress proteins. After 12 hours, normal synthesis in heat treated cells, was not dramatically depressed.

In general, total protein synthesis is more depressed under more severe stress conditions. Hickey [91] found that in HeLa cells, total synthesis was inhibited

for incubation at temperatures greater than 40°C (at 42°C, total synthesis was 20-30% of the rate at 37°C), but was not much inhibited at lower temperatures. Similarly, Levinson [122] noted that exposure to kethoxal bis(thiosemicarbazone) did not change overall protein synthesis in chick embryo cells at a concentration of 80ng/ml ( $2.8 \cdot 10^{-7}$ M), but that a concentration of 800ng/ml did inhibit overall protein synthesis.

### **Mechanism of Induction**

The agents which induce the stress response act on the cell or cell constituents by a number of mechanisms, but all either cause abnormal proteins to accumulate biosynthetically (either by errors in synthesis and processing of proteins or degradation), or are protein denaturants [92,17]. Recently, microinjection of denatured protein into cells was also shown to induce the stress response [51]. A number of investigators have suggested that the induction of stress protein synthesis is due to the accumulation of abnormal proteins [93,107,92,204,213,65,17] or changes due to catabolism of abnormal proteins [93]. Furthermore, a number have suggested that the stress proteins may play a role in the degradation of the abnormal proteins [93,125,65,51] or reformation of abnormal proteins [160,204]. Stress proteins have been ascribed a role in cell protection against the toxic effects of the various agents [160,40], and in allowing a return normal cellular metabolism after the stress has been removed [6]. Stress protein synthesis has been shown to protect against the toxic effects of various inducing agents. A low level stress (e.g. low temperature) protects against more severe stress in terms of increased survival (e.g. higher temperature) [6,2,127,198,161] or developmental defects (in *Drosophila*) [161]. Treatment by one inducing agent can provide protection, or partial protection against another [124,125,206,40]. The kinetics of thermotolerance correlate with the kinetics of stress protein accumulation or recovery [125]. Inhibition of stress protein synthesis inhibits return to normal synthesis [5]

Stress proteins may be involved in the reformation and degradation of proteins [160,51,65], and in this way protect cells against the toxic accumulation of abnormal proteins. Stress proteins have been shown to bind to other proteins. SP90 was found to bind to the viral product which mediates oncogenic transformation [26,38]. SP70 has been found to associate with a 90 kD cell surface glycoprotein [40]. Some stress proteins have been shown to be involved in ATP dependent protein degradation. The La protease in E Coli has been identified as a heat shock protein [17,40,65]. A small protein, ubiquitin, which has been identified as a stress protein [17,50,51] has been shown to form protein conjugates [87] and to be essential for degradation of short lived proteins in yeast [31]. Based on similarities between the phenotype of yeast defective in a gene coding for ubiquitin, and in a gene coding for SP70, Finley et al. suggest, that ubiquitin and SP70 may be functionally related [51].

In addition to a role in protection against toxic agents, stress proteins have also been linked to normal physical processes, especially cell growth and development. Stress proteins have been induced in sporulation in yeast [126,113], during development in *Drosophila* [150,40], and at the initial proliferation phase for PBMN in culture [105]. At very low levels they have been identified in chick embryo cells [106].

Synthesis of stress proteins can be induced under different growth conditions in cell culture. Synthesis of two stress proteins (80 and 100 kD) increases in mammalian cell culture under low glucose conditions [40]. Three stress proteins are induced in cell culture of *Tetrahymena* at high cell densities, coincident with high levels of intracellular glycogen. Certain of the stress proteins have been induced by exposure to platelet derived growth factor (chick embryo and rat embryo cells) [94] and by the hormone ecdysterone (in *Drosophila* [99]).

### **Stress Response in Chondrocyte Cell Culture**

Stress response has been induced in chondrocytes isolated from calf articular cartilage and in calf cartilage tissue culture, by incubation at elevated temperatures [132,131]. Stress proteins were also shown to be synthesized in chondrocytes isolated from human tissue by incubation at elevated temperatures and in chondrocytes isolated from human osteoarthritic cartilage at physiological temperatures [111]. Madreperla et al. [131] and Kubo et al. [111] identified two stress proteins, a 70 and a 110 kD protein, by gel electrophoresis (SDS-PAGE). On published gels, it appears that the intensity of two other protein bands, at 77 and 92 kD, increased a few hours following heat shock at 45°C. Thus, in cartilage, stress proteins are induced in response to at least two agents: heat, and the degenerative changes associated with osteoarthritis. Osteoarthritis is characterized by loss of proteoglycans, deterioration of the cartilage matrix, increased cellularity, and an increase in synthesis of DNA, proteoglycans, and collagen, and increases in some active enzymes which degrade proteoglycans and collagen [173]. Osteoarthritis is also associated with changes in the size of proteoglycans and their ability to interact with hyaluronic acid [29].

Madreperla et al. [131] and Kubo et al. [111] examined induction of stress proteins in cartilage in terms of dose response (the intensity of the response as a function of the temperature of incubation at a given temperature). The kinetics of the stress response in cartilage were also partially characterized.

The lowest temperature for which SP70 was detected on the gels in isolated chondrocytes isolated from normal tissue was approximately 39°C [132,111]. SP70 was induced in chondrocytes isolated from osteoarthritic cartilage at 37°C. SP110 was observed only inconsistently above 40°C [111] for 12 minute incubation periods for chondrocytes isolated from both normal and osteoarthritic cartilage.

The intensity of the SP70 band, for a 40 minute incubation at the chosen elevated temperature, increased between 37°C and 42°C, but was greater at 42°C than at 44°C [131]. The intensity of the SP70 band for a shorter (12 min) incubation

time, however, increased between 37°C and 44°C. The intensity at 44,45 and 46°C was approximately the same [111]. The intensity of SP70 induced in chondrocytes isolated from osteoarthritic tissue also increased for temperatures above 37°C [111].

Total protein synthetic rate in chondrocyte cell culture increased with temperature to 39°C, and then decreased monotonically between 40 and 45°C [132]. However, it is possible that this result might be an artifact of two different cell cultures. Chondrocytes isolated from one calf were used for temperatures between 34 and 39°C and chondrocytes isolated from another were used for temperatures between 40 and 45°C.

In terms of stress response kinetics, the work done by Madreperla et al. and Kubo et al. indicates that the time to induction of SP70 is less than a few minutes, that the persistence of SP70 synthesis during incubation at 37°C following incubation at elevated temperatures varies according to the duration of the heat shock, and that the half life of SP70 at 37°C is comparable to the half life of other proteins.

The time required for induction of SP70 synthesis in chondrocyte cell culture must be on the order of minutes (as in other cell cultures), since an incubation time of 12 minutes at temperatures as low as 39°C is long enough to produce observable SP70 synthesis, when labelled during or following treatment [111,131]. Similarly, the time required for SP110 synthesis is short, since SP110 is produced following a 12 minute incubation at 45°C.

Madreperla studied the persistence of stress response during incubation at 37°C following incubation at 45°C for 10, 20 or 30 minutes. The intensity of the SP70 band on gels did not increase with the time of incubation (between 10-30 min) at 45°C, indicating that the maximum response was induced by incubation for 10 minutes. Synthesis of SP70 was not examined as a function of duration of incubation at elevated temperatures for any other temperature.

However, while the intensity of the SP70 band did not increase with length

of incubation at 45°C, the persistence of SP70 synthesis in chondrocyte cell culture following heat shock, during incubation at 37°C, did increase with the length of incubation at 45°C. SP70 was synthesized for up to 5 hours following incubation at 45°C for 10 minutes, for up to 7 hours following incubation for 20 minutes, and for greater than 8 hours following incubation for 30 minutes. Actin synthesis (representative of normal protein synthesis) also returned more slowly following longer incubation at elevated temperatures.

Madreperla [132] reported that SP110 synthesis lags SP70 synthesis by an hour, following incubation at 45°C. Examination of the gel, however, suggests that the apparent lag may be due to the weakness of the SP110 band relative to the SP70 band, so that the SP110 band is not visible until nearly maximal synthesis of SP110 has been reached. Maximum intensity of SP110 occurred at the same time as maximum intensity of SP70. However, the intensity of the heat enhanced protein bands at 77 and 92 kD appeared to reach maximum intensity a few hours later, and their synthesis persisted longer than SP70 and SP110. In fact, these proteins did not appear more intense relative to controls on gels obtained from chondrocyte cultures that were labelled within 2 hours of the heat shock treatment. Kubo et al. [111] studied the half-life of SP70 in cartilage tissue culture. Cartilage tissue was incubated at 45°C for 12 minutes and labelled for three hours. The tissue was then washed and maintained in tissue culture. The tissue was digested to isolate the chondrocytes on designated days after heat treatment. He reported that SP70 had a half-life approximately equal to other proteins in normal cartilage tissue (11 days for adult tissue, and 7 days for young tissue), but that the half life in osteoarthritic tissue was much shorter (4-5 days).

### **Stress Response as a Marker for Field Effects**

The literature on the stress response in a variety of mammalian tissues suggests that stress response as a marker is sensitive and easily assayed by <sup>35</sup>S-methionine



incorporation followed by gel electrophoresis. Furthermore, the stress response is probably induced by agents that result in an accumulation of abnormal proteins. Thus, a variety of interaction mechanisms that result in changes in protein synthesis, processing, transport and degradation might induce this response. Furthermore, since stress protein synthesis has been associated with changes in cell development, it is possible that stress protein synthesis may be used as a marker not only for toxicity, but in addition probe for changes in cell state.

Specifically, as regards the protocol to test for electric field effect on tissues, the literature suggests that:

(1) It is necessary to test for a stress response to tissue excision, and if it is induced in cartilage tissue, to determine the length of time necessary to recover from excision, and eliminate stress protein synthesis.

(2) That the duration of stimulation and labelling should be a minimum of three hours, and the maximum time is not tightly constrained.

There appears to be no minimum time for heat to induce stress protein synthesis in cartilage tissue for temperatures above the threshold temperature. However, other studies (e.g. treatment with amino acid analogs) indicate that stress protein induction by other toxic agents may require some time (perhaps due to the time required to build up a pool of abnormal proteins). The longest time was on the order of a few hours. Therefore, the minimum time that cartilage should be electrically stimulated is on the order of three hours.

Lindquist [126] has suggested that synthesis of SP70 can be suppressed by accumulation of sufficient amounts of SP70. In response to incubation at elevated temperatures, stress protein synthesis has been shown to decline after several hours of continuous heat treatment. This might suggest that electrical stimulation should be limited to several hours to achieve maximum stress protein synthesis relative to normal protein synthesis. However, some studies of chemical treatments suggest that a high rate of stress protein synthesis is maintained for periods of greater than

12 hours. Furthermore, the work of Madreperla et al., which showed that SP70 synthesis in chondrocytes persists for up to 8 hours after incubation at elevated temperatures, suggests that times on the order of 8 hours or greater should not be excluded, particularly since stimulation of synthesis of the proteins at 77 and 92 kD appears to lag SP70 synthesis in chondrocyte cell culture. For electrical stimulation experiments (described in Chapter 3), cartilage tissue specimens were exposed to fields for a period of 12 hours.

(3) The work of Madreperla et al. suggests that ohmic heating to temperatures below 39°C may not induce stress protein synthesis in chondrocytes. Furthermore, since the kinetics of stress protein synthesis and the specific stress proteins induced depend on the inducing agent, so that it should in principle be possible to distinguish effects due to ohmic heating from direct field effects.

#### 1.4.3 Cartilage Tissue

Cartilage, specifically articular cartilage, was chosen to assess the effect of electric fields on tissues. Cartilage tissue is representative of a large and important class of body tissues, the connective tissues. These are characterized by a population of cells surrounded by a fluid filled extracellular matrix made up of primarily of collagen fibers and an amorphous ground substance of proteoglycans (PGs).

Cartilage was chosen in part because endogenous streaming potentials and currents are generated during physiological loading. Previous work has characterized the electromechanical properties of cartilage, as well as protein and GAG synthesis of cartilage in organ culture. In addition, a stress response has been shown to be induced in cartilage by heat, and may be important physiologically in joint articulation and osteoarthritis.

This section briefly reviews some background in cartilage morphology, as background for specimen preparation. Protein and GAG synthesis *in vivo* and *in vitro* are also reviewed in order to evaluate (1) maintenance of cartilage explants

in culture prior to electrical stimulation of the cartilage and (2) changes in protein synthesis in response to electrical stimulation. Details pertinent to the techniques to be used to assess stress protein synthesis ( $^{35}\text{S}$ -methionine labelling and SDS polyacrylamide gel electrophoresis (SDS-PAGE)) are emphasized.

### **Cartilage Morphology**

Articular cartilage is made up of a sparse population of cells, the chondrocytes (0.1-0.01 of tissue volume;  $10^6/\text{mm}^3$  in newborn human femoral condyle [192]), surrounded by a fibrillar collagen matrix of predominantly type II collagen, and a highly hydrated gel with glycoproteins (predominantly PGs and GAGs). Type II collagen comprises 65-75% of the dry weight of adult cartilage [76] or 20-30% of the wet weight in human articular cartilage [191]. The remainder of the wet weight is primarily water (up to 80% of wet weight [21,144], with PGs (approximately 20% dry weight [76]). The adult tissue is avascular and alymphatic, so that chondrocytes must obtain nutrients by means of diffusion from the synovial fluid surrounding the articular surface [138]. The synovial fluid is similar to plasma in low molecular weight components, and has relatively much lower concentrations than plasma in the high molecular weight components [195].

The structure and composition of articular cartilage varies according to the joint, age, individual, weight bearing history, and depth within an individual joint [112].

In adult articular cartilage, the variation in morphology with depth has been divided into four zones [112]: zone I—a superficial layer with the highest cell density, small discoidal chondrocytes (10-20 $\mu\text{m}$  in diameter in calf [57]), and collagen fibrils oriented parallel to the surface; zone II—an intermediate zone with more rounded chondrocytes (10 $\mu\text{m}$  in diameter) which are randomly distributed, and collagen fibrils that are oblique decussating; zone III—a deeper zone in which chondrocytes are larger and form columns perpendicular to the surface and collagen fibrils are

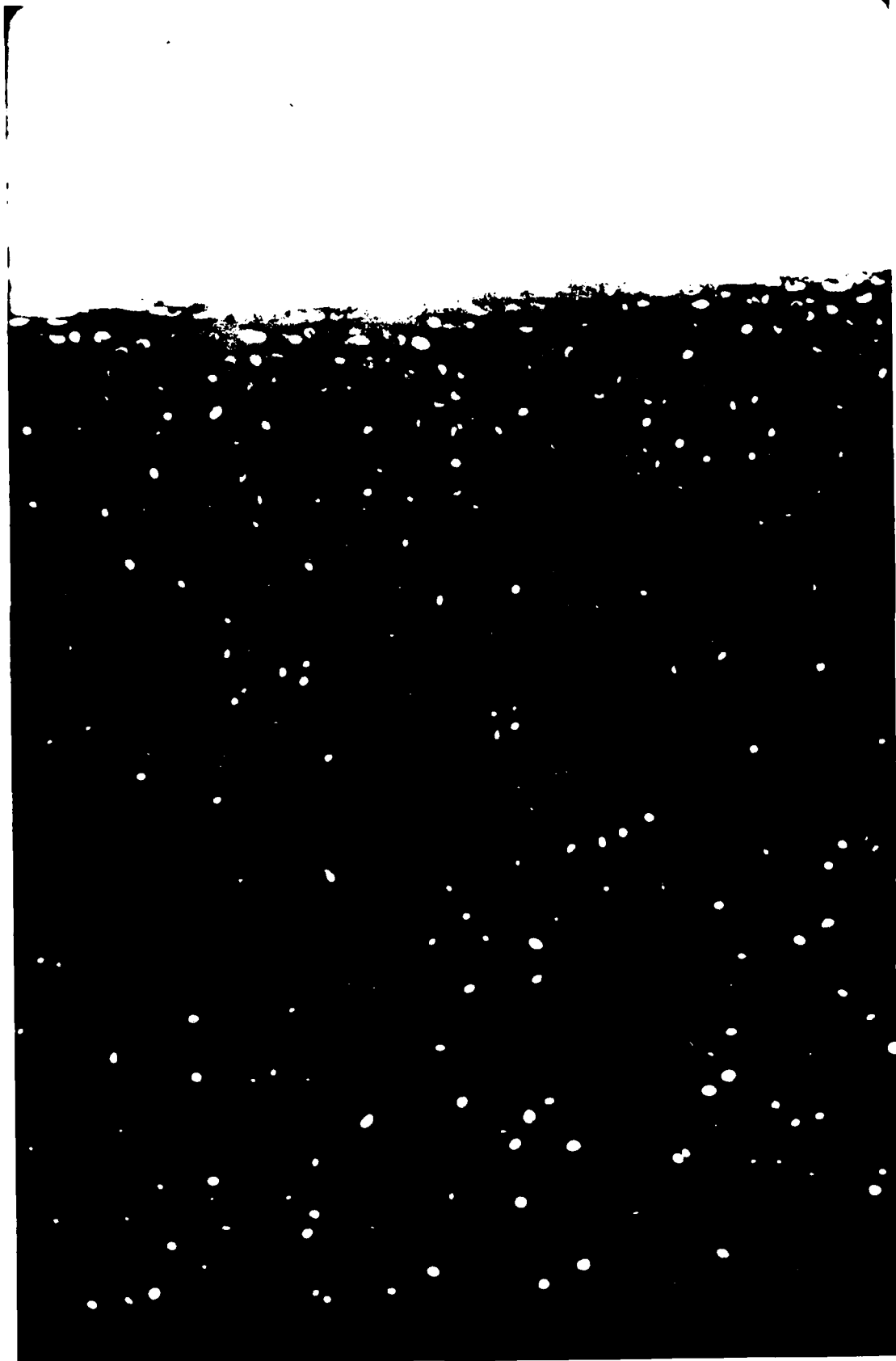
similarly oriented more perpendicular to the surface; and zone IV: the mineralized zone. PG content also varies with depth. In the superficial layer there is a lower PG content. In the deeper zone, the PGs have a higher proportion of keratan sulfate to chondroitin sulfate.

In young cartilage, such as the calf articular cartilage to be used in the experiments, zones II and III are a single zone in which there are small clusters of chondrocytes due to proliferation (diameter  $\approx 10 \mu\text{m}$  or more in calf [144], see Fig. 1.6). The number of mitoses varies with depth in immature cartilage [144]. In the developing epiphysis the number of mitoses is highest near the superficial layer and near the osseous center. Throughout zones II and III, the chondrocytes have no preferential orientation. Near the calcified layer, the chondrocytes hypertrophy but do not form columns.

Chondrocytes are surrounded by a thin layer of PGs, which is thicker in immature cartilage, and thickest in the deep zone ( $1\mu\text{m}$  [183]). Outside the PG layer is a fibrillar pericellular or territorial matrix comprised of thin fibrils, and in the bulk is the interterritorial matrix comprised of thicker fibrils. No observable difference in PG content has been found between the territorial and interterritorial matrix.

### Cartilage Biochemistry

A major function of chondrocytes in articular cartilage is to maintain the extracellular matrix (ECM) for load bearing in the joint. The extracellular matrix is comprised of type II collagen, and proteoglycans, as well as other matrix associated glycoproteins including the minor collagens ( $1\alpha$ ,  $2\alpha$ ,  $3\alpha$ , type IX and type X) and fibronectin and chondronectin. Turnover of collagen and PG is rapid in growing tissues, but is very slow in adult tissues. (Collagen turnover in human adult cartilage has been estimated to be 360 years, and in adult canine cartilage has been measured to be 120 years [192]). PGs turn over relatively faster, with two pools- a fast pool with half life on the order of a few days in young rabbit cartilage, and slow pools



**Figure 1.6: Histological section of a specimen of 1-2 week old calf articular cartilage**

on the order of a few months (50 days). Maroudas found that turnover times for PGs, similarly increase with age (16 days for 4 week rabbits, and approximately 300 days for mature rabbits [192]).

### **Type II Collagen**

Type II collagen, (and other collagens) is comprised of a triple helix of 3  $\alpha$  chains. The  $\alpha$  chains are made up of Gly-X-Y repeating segments. X and Y are often proline and hydroxyproline, which are required for triple helical formation [148]. (10 out of 1000 amino acid residues are methionine). In type II collagen, the  $\alpha$  chains are identical. The collagen monomers, are assembled into polymeric fibrils (average diameter  $\approx$  70 nm in articular cartilage [152], average length  $\approx$  335 nm in chick embryo sternum [100]), and in articular cartilage, these fibrils in turn form a loose meshwork which interacts strongly with the ground substance, primarily with the chondroitin sulfate (CS) chains of the PGs. The fibril diameter varies within the tissue— increasing from the surface toward the bone, from the perichondrium inward, and centrifugally from cells [152].

Collagens can be identified by SDS-PAGE (polyacrylamide gel electrophoresis), although they migrate anomalously, as if they were of higher molecular weight relative to globular proteins of the same molecular weight. SDS-PAGE of type II collagen shows primarily the collagen monomer. The collagen dimer appears only when the collagen is extracted with pepsin, or from lathrytic preparations [148]. The molecular weight of the collagen triple helix molecule is calculated to be approximately 290 kD [165]. The individual procollagen  $\alpha$  chains are 140 kD apparent molecular weight, and the collagen  $\alpha$  chain, post extracellular processing, is 95 kD [149].

Collagen is synthesized initially as prepro collagen. The N-terminal hydrophobic prepeptide probably binds to the membrane of the rough endoplasmic reticulum (RER) to lead the molecule across, and is removed after translocation [110,181] Proline and lysine residues are hydroxylated while the prepro collagen

molecule is being assembled, during translation and until formation of the triple helix. The hydroxyproline residues stabilize the triple helix under physiological conditions. The hydroxylysine residues stabilize intermolecular crosslinks and serve as attachment sites for carbohydrate links. The collagen molecule is glycosylated during assembly, and this continues until triple helix formation. Glycosylation may affect packing and collagen sensitivity to collagenases.

The propeptides may contribute properties necessary for transport and secretion of the procollagen molecule, and may play a role in fibril formation [147]. The N-terminal may act as a feedback inhibitor of procollagen [110]. The carboxy terminal propeptide probably serves as the initiation site for triple helix formation.

The site of collagen triple helix formation is not known, though Kivirikko [110] suggests that it is most likely either after the pro  $\alpha$  chains are transported to the RER, or as they leave the RER. The time required for triple helix formation in type II collagen is intermediate between type I (a few minutes) and types IV and V (an hour or more). The procollagen helices may then aggregate before leaving the cell via the Golgi complex.

Extracellular processing of the procollagen molecule includes cleavage of the terminal propeptides, fibril formation, and crosslinking of fibrils. Either N or C-terminal propeptides may be cleaved first, but for type II procollagen, the N-propeptides are most often cleaved first. Fibrils have been demonstrated to form spontaneously *in vitro* from procollagen helices. Proteoglycans, fibronectin, and other tissue components may affect the rate, length and diameter of fibrils formed. Crosslinks between fibrils are formed by means of lysine and hydroxylysine residues. These crosslinks become more stable over time. The crosslinking step is inhibited by lathrogens (e.g.,  $\beta$ -aminopropionitrile (BAPN)), so that culture in media containing BAPN can be used to obtain newly synthesized collagen molecules.

Approximately 1/2 the protein produced by freshly isolated chondrocytes is procollagen [110]. The rate of procollagen synthesis is affected by many factors



including: tissue type, growth, development, aging, hormones, peptide and protein factors [110]. The rates of intracellular processing and degradation depend on a similar array of factors.

### **Type II collagen C-propeptide**

The type II collagen C-propeptide (formerly designated chondrocalcin, due to its calcium affinity), has been isolated as a dimer of molecular weight 70 kD, which under reducing conditions subdivides into subunits of 35 kD (on SDS-PAGE) in bovine fetal epiphyseal and articular cartilage [170].

The tissue concentration of C-propeptide has been shown to increase with fetal age (in bovine fetal epiphyseal cartilage [30]), to concentrate in calcified tissues (bovine fetal growth plate cartilage [170]), and to vary under chondrocyte cell culture conditions [95]. Hinek et al. [95] investigated the variation in C-propeptide (CP) content during cell culture of chondrocytes isolated from bovine fetal growth plate, nasal, and epiphyseal plate cartilage in 10% serum (changed every 3 days). The ratio of CP to DNA content remained approximately constant from day 0-7 (decreased slightly in epiphyseal cultures only), while the CP content per culture increased from day 0-5, and then remained constant from day 5-7. In the epiphyseal cultures the ratio of CP to hydroxyproline (a measure of helical collagen content) increased between day 0-2, then decreased to a steady state between day 3-5. Hydroxyproline, normalized to DNA increased from day 0-1, then decreased to day 5.

### **1 $\alpha$ , 2 $\alpha$ , 3 $\alpha$ (Type K) Collagen**

1 $\alpha$ , 2 $\alpha$ , 3 $\alpha$  collagen, also designated type K collagen [134,149], is approximately 5-10% relative to type II content in cartilage tissue. It is comprised of three different  $\alpha$  chains. The 1 $\alpha$  and 2 $\alpha$  chains are similar to the  $\alpha$ 1(V) and  $\alpha$ 2(V) chains of type V collagen, and are not cleaved by mammalian collagenases [134]. The 3 $\alpha$  chain appears to be an over glycosylated form of the type II  $\alpha$  chain. Mayne and

Irwin [134] suggest that based on the resemblance of 2  $\alpha$  chains to type V collagen, and possible functional equivalence, that 1 $\alpha$ , 2 $\alpha$ , 3 $\alpha$  collagen may be involved in determining the diameter of type II fibrils.

### **Type IX Collagen**

Type IX has been shown by immunofluorescence techniques to be present in cartilage, and not in other connective tissues [100]. In cartilage, type IX collagen is 5-10% relative to type II [134]. The distributions of type IX were found to be the same as type II in chick embryo sternum, adult chicken and quail. Some immunofluorescent studies (in pig articular cartilage, epiphyseal calf, adult canine articular cartilage) found that type IX was located in the pericellular matrix. Irwin et al. [100], however, attribute this finding to masking of the collagen by PGs.

The function of type IX is unknown, but it has been suggested that it may be involved in the organization and stabilization of the type II structure [134].

Type IX collagen is a hybrid collagen, proteoglycan which is comprised of 3 different disulfide linked  $\alpha$  chains [205]. These form a collagen monomer with 3 helical domains (COL1, COL2, COL3), and four non-collagenous domains (NC1-4) [203,134]. The fibril length is  $\approx$  190 nm [100]. On SDS-PAGE, the intact, unreduced type IX collagen molecule isolated from chick embryo sterna runs at approximately 300 kD [205]. The individual chains, under reducing conditions run at approximately 84 and 61-69 kD, (chick embryo tibial cartilage) or at 115, 84 and 68 kD (chick embryo sterna). The chain of molecular weight 115 kD is apparently a 68 kD alpha core with CS chains attached to it, since digestion with chondroitinase, prior to SDS-PAGE, eliminates the 115 kD band, and increases the intensity of the 68 kD band [205]. The non-collagenous regions of the  $\alpha$  chains must be less than 10 kD, since digestion with bacterial collagenase eliminates all bands on SDS-PAGE [205].

### **Type X Collagen**

Type X collagen is comprised of 3  $\alpha$  chains of molecular weight 59 kD, which

are reduced to molecular weight 32 kD following collagenase digestion [134]. The 59 kD procollagen chain is reduced to a collagen chain of approximately 49 kD following extracellular processing [149]. The collagen molecule has a non-collagenous domain at the carboxy terminal. It has been suggested, based on the localization of type X collagen—its concentration increases toward the hypertrophic zone—that it may be a major protein in the hypertrophic stage [134].

#### **Fibronectin and Chondronectin**

Fibronectin (see [79] for a review) is a high molecular weight glycoprotein, located on the cell surface and in the pericellular and intercellular matrix. It consists of two disulfide bonded subunits ( $\alpha \approx 230$  kD and  $\beta \approx 210$  kD). In the matrix, it binds collagen and PGs, and forms self-aggregates by disulfide links.

Chondronectin, unlike fibronectin, is found exclusively in cartilage and in the vitreous body [79]. It is a 180 kD protein that is comprised of 80 kD disulfide bonded subunits, and acts to promote the interaction of cartilage cells with collagen.

Both can be extracted by a hot solution containing SDS.

#### **Proteoglycans**

Within the meshwork of the collagen fibrils, is the ground substance of cartilage comprised primarily of the highly charged proteoglycans and water. The high charge of the proteoglycans is responsible for the osmotic swelling pressures in cartilage which enable it to withstand compressional loads [76,47]. The proteoglycans are thought, in addition, to influence collagen fibril formation, growth and assembly into the three dimensional structure. The mechanism of action on structure is electrostatic [175]. By binding to charged groups on the collagen molecules, the PGs can stabilize the triple helix by increasing the melting point, and can influence the structure by changing the equilibrium of conformational states.

The proteoglycans are made up of a protein core with attached glycosaminoglycans, which in cartilage are predominantly chondroitin and keratan sulfate (approximately 100 CS and 50-60 KS per core in human adult [197]), grouped into

clusters along the protein core. Both structure and GAG content vary with depth from the articular surface. In the superficial layer, the PGs are filament like (8-12 nm diameter) and rich in KS. In the deeper layers, the PGs are leaf-like, and have a high proportion of CS [175]. Size, chemical composition and structure also vary in fetal development, and during maturation [174]. In immature cartilage, the monomers attached to the core protein are larger and more uniform in size, and the proportion of CS to KS is greater than in mature cartilage [174].

The proteoglycans form high molecular weight aggregates, by non-covalent interaction with hyaluronic acid and link protein. The link protein has been isolated from bovine epiphyseal tissue as 51 and 46 kD proteins (and a smaller amount of 44 kD protein in mature cartilage) [174]. Near the hinge region, at the site of link protein interaction, the PG molecule has a low GAG content which is primarily KS. The molecular weight of CS is approximately 20 kD, and of KS is much less. The proteoglycan molecule is approximately  $2.5 \cdot 10^6$  kD [197].

#### **Cartilage Explant and Chondrocyte Cell Culture**

Protein and PG synthesis in cartilage explant and cell culture has been shown to vary during culture, depending on the culture conditions [95,82,81,115,137,214,177,71]. Hinek et al. [95] investigated changes in procollagen synthesis in chondrocyte culture. Glycosaminoglycan synthesis has been reported to vary over the first 3-5 days in cartilage explant culture, and to depend on the culture media used [82,81,115,137]. Gray and Sah also found that both protein synthesis (assessed by proline incorporation) and GAG synthesis (assessed by sulfate incorporation) varied in calf epiphyseal and articular cartilage during the first few days of culture (see Appendix B).

Hinek et al. [95] showed that procollagen synthesis varies differently in chondrocyte cell cultures isolated from different tissues (bovine epiphyseal, nasal and sternal cartilage), over a period of days.

PG synthesis has been shown to vary with serum concentration and day of

culture. Hascall et al. [82] and Handley et al. [81] characterized proteoglycan synthesis in explant culture of adult steer and calf cartilage as a function of day in culture and as a function of serum concentration. In general, they suggest that cartilage metabolism in culture changes over a period of approximately 5 days, with the magnitude of the change depending on the specific tissue, on the culture method and on the culture medium used. Specifically, they found that for PG synthesis (assessed by  $^{35}\text{S}$ -sulfate incorporation), the concentration of serum (between 0 and 20 % fetal calf serum) in the culture medium determined the steady state level of synthesis achieved. At any time within 3 weeks, a change in serum concentration was able to produce a steady rate of synthesis that was nearly equal to the rate of synthesis achieved by culture in that concentration if used throughout the culture period [81]. In 0% serum, PG synthesis first increased (day 0-1), then decreased to a steady state lower than the day 0 rate. In 20% serum, PG synthesis increased monotonically to a steady state by day 5. The half life of the PGs synthesized also varied with serum concentration (approximately 11.4 day with 20% serum, and approximately 7 days with 0% serum). [81].

In 10% fetal calf serum, Lane and Brighton found a transient PG synthetic response in adult rabbit articular cartilage similar to that reported by Handley et al. in steer cartilage. for 20% serum (although absolute levels of synthesis at the steady state cannot be compared, since different cartilage was used, and Lane and Brighton used less frequent media changes than Handley et al.). In human articular cartilage, using 10% fetal bovine serum, however, McKenzie et al. reported a transient PG and protein synthetic response over the first few days that was more similar to the 0% transient reported by Hascall et al. and Handley et al. However, McKenzie did not extend his investigation beyond three days in culture. McQuillin et al. isolated an insulin like growth factor that mimicked the effect of serum on steady state PG synthesis.

Collagenase activity in serum-free culture of skin tissue (another type of

connective tissue) has been shown to vary with day after excision, with an increase in enzyme between days 2 and 4 [214]. Wooley suggests that this increase could be attributed to wounding effects of explantation, to adjustment to the new *in vitro* environment, to lack of *in vivo* restraints (e.g. hormones or plasma factors), or to an accumulation of enzyme, rather than production.

### **Protein Synthesis in Cartilage Tissue as an Experimental Model for Electric Field Effects**

The literature on cartilage morphology suggests that, in investigating electric field effects on cartilage, immature cartilage is preferable compared to adult tissue. Immature (e.g. calf) cartilage is preferable due to its greater homogeneity throughout the cartilage thickness (e.g. less variation in zones II and III). To enhance this homogeneity, removal of the superficial zone is indicated, as well as avoidance of the deepest layers near the calcifying zone.

The literature on cartilage biochemistry furthermore identifies a number of important proteins that might be identified by the SDS-PAGE techniques (e.g., separation according to molecular weight). These techniques are used to evaluate changes in synthesis of some specific proteins with applied electric fields (Chapter II). The apparent molecular weights associated with these proteins under reducing conditions are:

Type II collagen : pro $\alpha$  chain—140 kD ; C-propeptide—35 kD

Type IX collagen: pro $\alpha$  chains—86 and 64 kD

(5-10% of Type II content)

Type X collagen: pro $\alpha$  chains— 59 kD

(probably little contained in intermediate zones used for experiments)

fibronectin—230 and 210 kD

chondronectin—80 kD

PGs— $2.5 \cdot 10^6$  kD

The literature on cartilage biochemistry also shows that culture conditions and culture duration have have an important effect on synthesis, specifically total protein, PG synthesis [81,82,177,178,71], and procollagen synthesis [95]. Prior to electrical stimulation experiments, therefore, the effect of culture period on synthesis must be assessed for the serum concentration chosen.

#### 1.4.4 Review of the Literature on Electrical Stimulation in Cartilage

Mechanical deformation in connective tissues (e.g. cartilage and bone) gives rise to currents [54] and currents in turn give rise to mechanical stress [52,54]. Mechanical deformation has been implicated in bone remodeling [84,142] and changes in cartilage biosynthesis [71,73,44]. A question raised by the association of electric fields with mechanical deformation is: What is the biological effect of an applied electric field on connective tissues? A variety of cell and tissue responses to applied electric and magnetic fields have been documented. Investigators have used a wide range of stimulation amplitudes and frequencies in the study of field effects *in vitro* and *in vivo*. In cartilage, electric fields have been reported to stimulate DNA synthesis in isolated epiphyseal [172] and articular [23] chondrocytes, enhance cAMP accumulation in intact epiphyseal cartilage [156], increase GAG synthesis in isolated chondrocytes [190,23] enhance proliferation of differentiated chondrocytes [154], and to stimulate accelerated growth of rabbit tibial growth plate. Application of pulsed magnetic fields has been found to increase GAG synthesis and decrease collagen and total protein synthesis [3].

These studies do not, however, form a cohesive framework in which to understand the effects of electric fields on connective tissues. Partly, this lack of cohesion is due to experimental designs which do not permit effective comparisons to be made between the results reported by the different laboratories. Some important parameters have not been well defined in experimental protocols, including: electric field magnitude or current density, electrode effects, temperature, and control pairing. In addition, there are differences in the tissue models, and the applied field amplitudes and frequencies differ between studies. Furthermore, the frequencies and amplitudes, are not in general motivated by considering amplitudes or frequencies at which certain physiological mechanisms might be important, or by comparison with physiologically generated field magnitudes and frequencies, but more by practical clinical considerations. Application of electric or magnetic fields for clinical use



has demanded the development of noninvasive techniques. Therefore, for clinical purposes, frequencies are chosen to produce field magnitudes or energy densities within the tissue using inductive or capacitive coupling.

The frequency of stimulation is an important consideration in the understanding of the mechanism of interaction with applied fields, as indicated previously in this chapter. Electric field stimulation frequencies have included dc [207,22], sinusoidal fields between 0.1 - 100 Hz [71], and 60 kHz [23], and pulse waveforms with a 1 Hz [207] or 5 Hz [156] repetition rate. Pulsed magnetic fields have used a variety of complex waveforms [3].

In most of the electric field stimulation studies, the maximum amplitudes of the electric field or induced current density at the site of the cartilage tissue or cell preparation can be estimated to be within the range of  $10\text{-}1000\mu\text{A}/\text{cm}^2$ . The complex geometry of the specimen preparation, the complex methodology for application of the field, and in some cases, the complex waveform of the applied electric or magnetic field makes it difficult to quantify the exact field magnitude induced within the tissue. With non-invasive capacitive and inductive coupling systems, the electric field at the site of interest depends on the frequency, position of the source (i.e., electrode plates or coils), the complex tissue dielectric and conductivity properties, and the interfacial boundary conditions within and between different tissues. The *internal* fields, those most relevant to a field dose response, therefore, are generally not known. Because of the many different stimulation amplitudes and frequencies used, it is often difficult to compare the results of the studies found in the literature.

In vivo models, have naturally complex geometries. For example, Brighton et al. [24] and Armstrong and Brighton [4] stimulated the tibial growth plate of adolescent rabbits by means of capacitor plates attached over the rabbit proximal tibiae. Brighton et al. used 60 kHz for 48 hours with 2.5, 5, 10 and 20 V p/p across the capacitor plates. Growth was assessed by the distance moved by an

oxytetracycline label into the metaphysis during stimulation. They reported that growth was enhanced by field stimulation with an applied voltage of 5 V. Armstrong and Brighton [4] stimulated the tibial growth plate using 60 kHz at 5 V continuously for 39 days. Growth was assessed by the distance moved by a tantalum bone marker insertion. No stimulated acceleration of growth was observed.

The complex boundary conditions on the field in combination with the complex *in vivo* geometry make current and voltage regulation difficult, as pointed out by Brighton et al. [24]. At a given frequency, and with identical electrode and tissue geometries, however, the voltage and current should be linearly related. In practice, this is not always the case [24], presumably due to changes in electrode configuration or boundary conditions (e.g., skin impedance). As a result, at a single frequency of interest, changes in applied voltage may not lead to changes of the same magnitude in the field at the site of interest. Brighton et al. [24] measured the mean capacitor currents corresponding to the voltages applied across the capacitor plates. These were:

2.5 V : 1.24 +/- .43 mA

5 V : 1.19 +/- .42 mA

10 V : 1.53 +/- .64 mA

The measured total currents are within one standard deviation of each other, while the voltages have increased fourfold. Furthermore, the current does not increase for an increase in voltage between 2.5 and 5 V. Since the parameter of interest is the internal field magnitude, the question is which measurement (voltage or current) more accurately represents the internal field magnitudes. The answer would depend on the reason for the non-linear relation between the voltage and current. It could be due to differences in interfacial resistance at the electrode or to differences in electrode contact or contact area. It could be argued that internal interfacial conditions would not differ by much, since for a given frequency, internal tissue conductivities and dielectric constants are expected to be similar from animal to

animal, and the distribution of current flow within the leg must also be similar. In the above experimental example, then, if the difference in current for a given applied voltage is due primarily to differences in voltage drop (interfacial resistance) at the electrode interface, then continuity of current would imply that the *magnitude* of the *current* measured is proportional to the magnitude of the internal field. On the other hand, if the electrode interface *area* changed, then the current distribution within the tissue will be altered, and the total current may not be proportional to the magnitude of the internal field. In the absence of information on local field magnitudes, interpretation of experimental results is difficult. In Brighton's study, for example, if the total current, rather than voltage, is proportional to the internal field magnitude, then it does not make sense to have a response at 5 V and not at 2.5 or 10 V since the currents at these voltages are within a standard deviation of each other.

Determination of internal field magnitudes becomes even more uncertain when more than one frequency is used. In related fracture healing studies, for example, Brighton et al. [23] varied both voltage and frequency. Interfacial tissue and electrode interfacial boundary conditions, as well as bulk electrical tissue properties depend on the frequency, so that a change in frequency affects the field *distribution* as well as magnitude within the tissue. It is not possible, therefore, to compare results from multiple frequencies in terms of the field magnitude at the site of interest, since neither the magnitude of voltage, nor the total external current, will necessarily reflect the magnitude of local internal fields.

Experiments in culture may also have dosimetry problems as difficult as those in animal or clinical tests, unless tissue geometry and the electrode geometry for applying the field are chosen specifically to simplify field calculations. For stimulation of whole tissue in culture by capacitive coupling, for example, the field at the tissue depends on boundary conditions at the culture dish interface and media (and so depends on media depth), as well as on the electrode geometry.

In some tissue culture configurations, field magnitude has been miscalculated. Watson et al. cultured embryonic chick tibia in petri dishes between two plates 1.4 cm apart, and stimulated the tibia with a d.c or with a 1 Hz pulsed field of 1000 V/cm. They reported that dc fields did not stimulate bone growth, as assessed by measuring the fractional increase in the bone length relative to matched controls, but that pulsed electric fields did promote bone growth.

Brighton et al. [22], cultured rat costochondral junction in media in petri dishes between two capacitor plates, and stimulated for 10 days using d.c. field magnitudes of up to 3000 V/cm. They assessed growth by measuring the fractional increase in length, and incorporation of  $^{45}\text{Ca}$ ,  $^{35}\text{S}$ -sulfate and  $^3\text{H}$ -thymidine. Elongation was reported to respond to electric field in a dose dependent manner, with a maximum response at 1500 V/cm. A significant increase in incorporation of all three radioisotopes was reported for an applied field of 1500 V/cm.

In both these experimental configurations, however, the capacitively coupled dc fields will not induce local electric fields at the site of the tissue of interest. Induced charge at the media to air or plastic interface shields the interior of the media from any electric field. (This surface charge changes in response to time variation in the external field, so that, in the case of time varying fields, current does flow through the media.) The results reported by Brighton et al., therefore, are not consistent with an applied field effect, since no field was present at the tissue site. The results reported by Watson et al. are consistent with the field theory, in that no effects are reported for d.c. fields (no field induced at the tissue site), and effects are reported for a time varying field, (fields induced within the tissue). However, the magnitude of the induced time-varying field is unknown.

The same capacitive coupling configuration, was used appropriately for higher frequency fields [23]. Brighton et al. stimulated chondrocytes isolated from bovine articular cartilage using 60 kHz for 24 hours. The culture dish was filled in order to eliminate a media to air interface, and the dish fit tightly between two

capacitor plates. Biosynthesis of GAGs and DNA was assessed by incorporation of  $^{35}\text{S}$ -sulfate and  $^3\text{H}$ -thymidine in culture, normalized to total DNA and protein content. They reported no change in sulfate incorporation by cultures in 10% newborn calf serum (NBCS) and an increase at 250 V/cm by cultures in 1% serum, a decrease in  $^3\text{H}$ -thymidine incorporation by culture in 1% serum (normalized to DNA content), and an increase at 250 V/cm by cultures in 10% serum.

Experimental geometries, therefore, lead to field magnitudes that are not defined at the tissue site. Similarly, stimulation via waveforms that are complex in terms of frequency components, such as pulsed waveforms have led to problems of local field dosimetry. Boundary conditions at all interfaces depend on frequency, so that internal field magnitudes are the sum of the contribution to the local magnitude by each component frequency of the stimulation waveform.

Norton et al. [156], for example, stimulated using a 5 Hz,  $10^{-5}$  second pulse, so that at least two frequencies are of interest—5 Hz and 100 kHz. Curved electrodes were fitted to the outside of tubes containing a chondrocyte suspension, or about tibiae oriented at right axes or parallel to the field. In each case cAMP accumulation was assessed by radioimmunoassay following stimulation. They report an increase in cAMP accumulation in bone at fields above 600 V/cm, and a decrease in cAMP accumulation in chondrocyte suspension.

Archer and Ratcliffe [3] stimulated chick embryo tibia via pulsed magnetic fields with an exponential rise and decay. Pulsed electromagnetic fields generated by coils from Electro Biology Inc., which also have a complex waveform, have been used in a number of studies on bone growth and remodeling [190].

Direct ohmic electrode connections can facilitate the control of field magnitude at the site of interest. Gray et al. [71], for example, stimulated cylindrical disks of calf epiphyseal cartilage in a chamber filled with culture media using platinum electrodes located in the media at the ends of the chamber. The experimental chamber was designed so that current continuity defined the magnitude of the cur-

rent density (field magnitude) within the sample. The cylindrical disks were held in place in a cylindrical hole perpendicular to current flow, such that the current was constrained to flow through each sample in series. Thus, the construction ensured that the current density was uniform across the cross section of the sample. Gray et al. [71] assessed changes in biosynthesis in stimulated cartilage disks by measuring incorporation of  $^3\text{H}$ -proline and  $^{35}\text{S}$ -sulfate. No effect on synthesis was reported for current densities (local densities within the sample) of up to  $1 \text{ mA/cm}^2$ , and frequencies of 0.1 to 100 Hz.

One drawback to stimulation by direct ohmic coupling is that current flow can generate electrode reaction products that can artifactually alter cell behavior. Current flow can generate changes in pH ( $\text{H}^+$  generation), oxidizing agents, and gas ( $\text{O}_2$ ) formation at the anode [27], and pH changes ( $\text{OH}^-$  generation) and gas ( $\text{H}_2$ ) formation at the cathode. Metal oxidation products specific to the electrode metal can also be produced at the cathode (e.g.  $\text{PtCl}_6$  is produced at a platinum anode). These products are known to be biologically active [27].

For certain frequencies and very low current densities, generation of significant irreversible electrode products may be small enough to be unimportant. pH changes produced by electrolysis at platinum electrodes may be small enough to be compensated for by the buffer in the culture medium. Lee et al. [118], for example, stimulated chondrocytes isolated from chick embryo sternae and cultured on elastin membranes, via stainless steel electrodes within the culture media. Electrodes were located 2 cm from the membrane so that electrode products would not diffuse to the membrane site within the stimulation period. No changes in pH were measured, for current densities of  $10^{-4}$  to  $0.1 \text{ mA/cm}^2$ . Gray [71] used a long media bridge between the electrode and tissue sample to eliminate possible electrode artifacts.

However, even moderate current densities can generate significant electrode reaction products if care is not taken. In initial experiments done for this thesis, for example, protein synthesis was altered in cartilage samples that were stimulated by

ac currents (at 1, 10, 100 and 1 kHz). This change was subsequently identified as due to electrode reaction products (Chapter 3).

The problem of electrode reaction products is likely to be most acute for dc stimulation. At dc, the only mechanism for current flow is irreversible ion generation at the electrodes. In the case of ac currents, at low enough current densities, reversible electrode reactions can keep chemical changes to a minimum. Nogami et al. [154] used electrodes connected directly to the tissue of interest to apply dc fields. Minced muscles from rat fetuses cultured in bone matrix gelatin hemicylinders were stimulated via platinum electrodes at the ends of the explants. Explants were stimulated using a total current of 5  $\mu$ A on days 1-7, 8-14, and 15-21 of culture. Field induced changes in cartilage formation were assessed by fixing the specimens, staining with methylene blue and counting the number of crevices with cartilage formation. Poor cartilage formation was reported in the anode and cathode region in the first week, and in the anode region, in the second week. The inhibition of cartilage formation is likely due to electrode products. The current density at the electrode used by Nogami et al. can be estimated, based on the electrode diameter to be not lower than .01 mA/cm<sup>2</sup>, and might be as high as 20 mA/cm<sup>2</sup>. The localization of the reported changes to the region near the electrodes further supports the interpretation that the effects are due to electrode reaction products.

Electrode reaction products, therefore can produce artifactual changes in biosynthesis that are not due to a direct field effect. General or local tissue temperature rises produced by electrically induced ohmic heating can also produce biosynthetic responses that are not a direct field effect. Heating can affect overall cell behavior (through an increase in reaction rates), or a more specific response (such as the production of a specific set of proteins: the stress proteins [185]). The issue of field induced heating, therefore, is critical in any study of field effects on biosynthesis. In none of the studies cited above, however, with the exception of those done by Gray et al., was temperature measured or controlled, so that ohmic

heating cannot be eliminated as a possible artifact. Current densities, however, are generally estimated to be small enough not to produce significant ohmic heating.

Control studies and statistical analyses can be important in evaluating the significance of reported field effects. In particular, proper pairing of stimulated and control samples and careful normalization can reduce scatter and produce more meaningful results. Matching tissues from the right and left sides as the experimental and control pairs is one method that has been used in tissue culture [207,172]. Pairing cell cultures from the same tissue has also been used [172], or tissue samples from the same tissue [71]. Whole animal studies pose particular problems of matching. Approaches to the problem of matching animal populations have included choosing a population that had demonstrated a similar weight gain prior to exposure [4] matching populations by day of mating [190]. Methods of matching experimental and control populations, however, have not always been recorded [156,154,24]]. In the study done by Brighton et al. [24], for example, the question of matched animals is particularly critical. In this study the ratio of right to left tibia elongation was compared for animals receiving the electrical stimulation relative to animals wearing the same apparatus but without stimulation. Brighton et al. reported that the right to left elongation ratio was significantly greater in the group stimulated by 5 V, relative to its control group. However, the ratio in the experimental group is similar to ratios in the other experimental groups (1.19 compared to 1.21, 1.24 and 1.12), whereas the ratio for the control population is less than that of the control populations corresponding to the other experimental groups (1.09 compared to 1.15 and 1.16). The statistical significance of the effect at 5 V/cm, therefore, is a consequence of a lower ratio for the control population, rather than a higher ratio for the experimental population. In the absence of a stated rationale for matching experimental to control populations, the real significance of the enhancement of growth by the 5 V/cm field is somewhat suspect.

Multiple treatments (stimulation protocols) using a single control popula-



tion may lead to erroneous interpretation. Nogami et al., for example, stimulated 4 groups of specimens, of which one served as a control population. The standard deviation in results and numbers of specimens per treatment is not given, and variation within the control population is unspecified. Furthermore, the statistical analysis is based on the t test, which is not valid for comparison between more than 2 treatments. Brighton et al. also compared 3 stimulation treatments (at 10, 60 and 250 kHz) to a single control population. The 60 kHz and control stimulation were apparently run concurrently, while the 10 and 250 kHz stimulation was done subsequently. Yet these latter treatments are compared to the same control population.

In summary, a review of the literature on field effects on connective tissue biosynthesis and growth has demonstrated that such effects are not always well defined due to undefined internal field magnitudes, possible electrode and temperature artifacts, and in some cases, inadequate controls. Furthermore, the large variation in experimental stimulation frequencies used, makes it difficult to construct a framework within which the reported results can be understood.

### 1.5 Summary

In this chapter the magnitudes and frequencies of endogenous and exogenous fields have been estimated as an indicator, respectively, of the range of magnitudes and frequencies that may be important to tissue or cell physiology, and of the range of applied fields which might act on tissues. The magnitude of endogenous fields ranges from  $1\text{-}1000\mu\text{A}/\text{cm}^2$  or higher, and the frequencies from dc-1 kHz. The frequency range of known exogenous fields is much larger, from dc to approximately 100 GHz. The magnitudes of internal fields induced by external environmental sources (based on the ANSI standards), might be on the order of endogenous fields for frequencies

above 1 MHz. Below a few kHz, induced internal field magnitudes decrease linearly with the frequency, so that internal fields induced by environmental sources will be smaller. In clinical applications, however, such as the use of electric fields to accelerate healing of connective tissues, internal fields might again be on the order of endogenous fields.

Several possible mechanisms by which induced internal fields might interact with tissue have been identified. The magnitude of the changes that could be produced via these mechanisms were estimated and characterized in terms of the frequency regime in which they might be important. Specifically, a 1-10% change in transmembrane potential could be induced by applied fields on the order of endogenous fields ( $1 \text{ mA/cm}^2$ ) for frequencies less than 10 MHz. A 1-10% change in intramembrane concentrations could be induced, by the mechanism of electrodiffusion across a membrane with the dimensions of a cell membrane (10 nm), for frequencies below 10 MHz only for very large fields. Such large fields (a 10% change of the cell transmembrane potential is equivalent to a field magnitude of  $10^6 \text{ V/m}$ ) can be induced across the membrane, however, by a field external to the cell, for frequencies much below 1 GHz, as discussed above. A 1-10% change in concentration across a much thicker region, such as connective tissue matrix requires a much smaller field magnitude ( $\approx 100 \text{ V/m}$  for a  $10 \text{ }\mu\text{m}$  thick membrane), but the corresponding frequency limit determined by the electrodiffusion time is also correspondingly lower (1 Hz for a  $50 \text{ }\mu\text{m}$  thick region). The resulting ac transmembrane flux has a small (1%) time average component. A 1-10% change in pore size, based on the double layer model, could not be induced except by very large fields, and the kinetics of response to ac fields are not known. Field driven movement of macromolecules (within the intra or extracellular space, or on the surface of the cell membrane), at the field strengths considered, might be important only for the case in which the small distances travelled in one period (e.g. nm for a 1-10 V/cm and 1-10 Hz field) are significant. Such a case might be motion of molecules along

the surface of a cell membrane). Thus a significant ac change in transmembrane potential might be induced by fields below 10 MHz. Subsequent changes in ion flux (by the mechanism of electrodiffusion) might be important below 10 kHz. Changes in concentration and ion flux across thicker membranes require lower frequencies. Finally field driven electrophoretic motion on the cell surface is likely to be important only below 10 Hz. Thus, it appears that the range of frequencies for which an applied field might act on cells and other tissue components is between dc and 10 kHz.

Modulation of stress and total protein synthesis in cartilage tissue was proposed as a model for examining electric field effects on biological tissues for fields within this range of frequencies, and magnitudes within the physiological range (e.g. on the order of mA/cm<sup>2</sup>). Stress protein synthesis is sensitive, and is induced in response to a variety of agents which act to produce an accumulation of abnormal proteins. While induction of stress protein synthesis is not mechanism specific, the particular kinetics and the particular stress proteins induced are specific to the inducing agent. Changes in normal protein synthesis is part of the stress response, but in addition, changes in synthesis (in the case of cartilage specifically) occur in response to other changes in state (e.g. in the response to organ culture, or to mechanical stimulation [71,179]).

Stress protein synthesis depends both on tissue type and on culture conditions. Stress proteins have been induced in chondrocyte cell culture, and partially examined in response to heat, as described earlier. However, in order to use stress protein synthesis as a probe for electric field effect, it is clear that the stress response must be characterized for the particular tissue and culture conditions used.

Some characteristics of normal protein synthesis in cartilage, including the major proteins produced by the chondrocytes, and changes observed during culture have been described. Clearly, normal protein synthesis in the particular cartilage tissue culture conditions used prior to electrical stimulation must also be charac-

terized. Control studies to characterize stress protein synthesis and normal protein synthesis are discussed in the chapter following.

The literature on electric field effects on intact cartilage tissue or chondrocyte cell culture indicates that electric fields may change the biosynthetic behavior of cartilage, but does not provide a coherent framework for either predicting the effect of any particular field or frequency, or for understanding the mechanism of action. Some requirements for developing an experimental framework for the effect of electric fields on cartilage tissue are indicated. These include: a well defined field magnitude and frequency within the tissue, (achieved in this study by using ohmic coupling and a simple uniaxial geometry); temperature measurements (measured in this study, by thermistors adjacent to the specimens); controls for electrode reaction products (agar plugs were used to isolate the specimens from the electrodes, and control studies demonstrated that these were effective); and adequate controls (achieved here by characterizing the biosynthetic behavior of the specimens prior to stimulation, and matching specimens with *similar biosynthetic rates*).

## Chapter II

### Experimental Model: Control Studies

#### 2.1 Introduction

In the previous chapter, it was suggested that stress protein synthesis and total protein synthesis are appropriate probes for investigating the effect of electric fields on biological tissues over a wide range of frequencies and amplitudes. Over this range several physical mechanisms might be important. Stress protein synthesis is an indicator of toxic effects (and possibly other changes in state) that is not mechanism specific. As discussed in chapter I, changes in synthetic rate of GAGs [177,178,72,115,81,82], and proteins [177,178,72,95,143] during cartilage organ culture have been reported previously. Synthesis of proteins other than stress proteins, therefore, can additionally mark changes in metabolic state which are not necessarily related to a toxic stimulus.

Cartilage is an appropriate choice of tissue. Streaming currents are generated as part of its normal physiological function. Articular cartilage has been previously characterized in terms of total protein and GAG synthesis, and variation in synthetic rates across the joint surface. The conductivity of cartilage is nearly that of physiological saline, which facilitates field calculations. Finally, stress protein synthesis has been observed previously in cartilage [132,131,111].

Chapter II and Appendix B detail the control studies that were used to establish the biochemical protocols to assess stress protein and total protein synthesis and the culture conditions, and to evaluate changes in protein synthesis produced by electric fields.

To assess protein synthesis, cartilage plugs were incubated in media containing  $^{35}\text{S}$ -methionine. Following the chosen labelling period, proteins were extracted from the plugs in two fractions, an SDS-soluble extract and a papain digest. The SDS-soluble extract consists of intracellular proteins and extracellular matrix pro-

teins that have not been incorporated into the matrix and are able to diffuse out of the tissue. The papain digest fraction consists of extracellular proteins that have been incorporated into the matrix, or are unable to diffuse out. Total radioactivity in each fraction, which represents the incorporation of label into each fraction, was measured by scintillation counting. The SDS-soluble fraction was run on a polyacrylamide gel (Appendix B) to separate and visualize individual proteins, especially the stress proteins.

Culture conditions were established with the aim of maximizing the sensitivity of stress protein and total protein synthesis to electric field stimulation, with the constraint that protein synthesis prior to field stimulation must be consistent, that is, in steady state, between experiments. With steady state levels of protein synthesis, results from a series of electrical experiments can be compared. Therefore, a time in the culture period during which protein synthesis was in steady state for a period of several days was identified. Cartilage plugs were exposed to electric fields during this time in the culture period, so that synthesis in these plugs had reached steady state prior to field exposure. Culture parameters which have been shown to have an important effect on protein synthesis in cartilage tissue culture include the concentration of serum in the incubation medium and the length of culture (Chapter I, Cartilage Biochemistry).

Previous control studies [71,177] suggested that organ culture using a serum concentration of 0.1% NuSerum (NS) would be appropriate conditions for assessing changes in total protein synthesis. Cartilage plugs maintained in DMEM with 0.1% NS were shown to reach a steady, but neither maximal or minimal rate of synthesis after a few days in culture (Appendix B.6). Thus, the tissue is at an intermediate level of activity and has the metabolic capability to increase or decrease synthesis in response to electric fields. In this chapter, further controls are described which showed that in 0.1%NS both incorporation in the SDS-soluble extract, and in the papain digest reached a steady level after approximately 4 days in culture. The

intensity of some specific protein bands visualized on the gels changed following day 4. Therefore, to do electrical experiments under consistent conditions, a 5 day culture period was used prior to electrical stimulation of the cartilage plugs.

In contrast, plugs cultured in 10% fetal bovine serum (FBS), had a high steady level of protein synthesis, relative to plugs cultured in 0.1%NS after three days in culture. Culture in 10% FBS, therefore, would appear to make total protein synthesis a less sensitive probe for stimulatory effects of electric fields. Plugs incubated in 10% FBS did not show any apparent changes in intensity of specific proteins on gels.

Heat was first used as a control stimulus to induce the stress response in order to:

- (1) confirm that the biochemical techniques (especially the labelling and SDS-PAGE) were sensitive enough to detect the stress response;
- (2) identify culture conditions for maximum sensitivity to stress (heat or electric fields); and
- (3) examine the stress response to heat so that field induced effects could be compared to heat induced effects.

The controls showed that the biochemical techniques were indeed effective in detecting stress protein synthesis. A severe heat shock, incubation at 43°C for 6 hours, identified the heat inducible proteins. The intensity of the stress protein bands on the gels depended on the temperature and on the duration of incubation at elevated temperatures.

Culture conditions that were indicated for using the stress response as a marker were similar to the conditions for using total protein synthesis as a marker for electric field effects on cartilage tissue. Culture for several days using a serum concentration of 0.1%NS produced a high level of stress protein synthesis in response to heat, no apparent stress protein synthesis in the absence of heating, and a distinct change in total protein synthesis in heated specimens relative to controls.

The intensity of two stress protein bands induced by heat (SP70 and SP78) appeared to be greater relative to the intensity of normal protein bands for cartilage plugs incubated in 0.1%NS compared to cartilage plugs incubated in 10% FBS, throughout a culture period of nine days. Thus culture in 0.1%NS rather than 10% FBS was indicated for visualizing stress proteins in response to a stimulus such as heat or electric fields.

The controls studies indicated that the optimum length of culture in 0.1%NS was greater than 5 days. First, SP70 was induced in cartilage plugs incubated at 37°C on earlier days. Second, the intensity of the stress protein bands induced by heat appeared to increase over the first few days. Finally, the change in the ratio of radiolabel incorporated into the SDS-soluble fraction relative to the papain digest, which accompanied induction of stress proteins by heat, also increased after 4 days of culture. Therefore, in order to eliminate stress protein synthesis in the absence of a stimulus, and to obtain the greatest change in stress protein and total protein synthesis in response to a stimulus, cartilage plugs were cultured for a minimum of 5 days.

The stress response was examined in terms of the intensity of stress protein induction as a function of temperature and duration of incubation at elevated temperature, and in terms of the accompanying changes in total protein synthesis. The intensity of SP70, the major stress protein, appeared to be maximally induced for long exposures at 43°C. The intensity generally increased with the duration of incubation, up to a maximum determined by the temperature of incubation. The intensity of SP70 appeared to increase with temperature (at least between 41 and 43°C). Stress protein synthesis was not induced below 39°C. This is important, since it means that in electrical experiments stress proteins would not be induced by ohmic heating, until a few degree rise in temperature. Heat induced stress was accompanied by changes in total protein synthesis. In particular, the stress response to incubation at elevated temperatures was accompanied by an increase in



the amount incorporated into the SDS-soluble fraction relative to the papain digest. This again is important, since as shown in Chapter IV, the change in ratio distinguishes between heat induced changes and field induced changes in protein synthesis.

In conclusion, the controls studies described following indicated that for using total protein and stress protein synthesis as a probe, optimum culture conditions consist of a low serum concentration (0.1% NS) in the culture media, and an incubation period of 5 days prior to the application of an electrical or thermal stimulus. Plugs were cultured for no more than 8 days prior to electrical stimulation in order to remain within the culture period investigated in the control studies. The response to heat in terms of stress protein and total protein synthesis was examined, so that it could be compared to any field induced changes in protein synthesis.

## 2.2 Methods

The culture, labelling and biochemical protocol followed routinely in the electrical stimulation experiments, was based on a series of control experiments detailed in Appendix B. These included:

(1) Comparison of a collagenase digestion and SDS extraction protocol to obtain proteins from cartilage specimens for gel electrophoresis (Appendix B.1). Extraction of proteins by boiling plugs in SDS sample buffer for gel electrophoresis was then used routinely since this method was simple and produced consistent results. Furthermore, it enabled the measurement of total incorporation into two protein fractions which together included both intracellular and extracellular proteins.

(2) Measurement of radiolabel incorporation as a function of the concentration of cold methionine added to the media and the concentration of  $^{35}\text{S}$ -methionine. The objectives were to maximize the specific activity and to define the total methionine concentration in the labelling media.

Some added cold methionine in the labelling media was required to define the concentration of total methionine in the labelling medium. Cartilage plugs transferred from culture medium with 2 mM methionine to labelling medium without methionine contained some cold methionine in the plug fluid volume, so that the total concentration of methionine in the labelling media was determined by this residual methionine (Appendix B.4). Incorporation was inversely proportional to the concentration of cold methionine added for greater than 0.1 mM added methionine. Therefore, 0.1 mM cold methionine was routinely added to the labelling medium, so that the total methionine concentration within the labelling medium was well defined. (This also eliminated the possibility that methionine deprivation might in itself induce a stress response [93], although this has not been observed.)

Incorporation was proportional to hot methionine concentration (Appendix B.3) for an added cold methionine concentration of 0.1 mM. Therefore, hot methionine concentration in the labelling media could be changed, if necessary, and total incorporation normalized to the hot methionine concentration.

(3) Determination that a minimum 1/2 hour chase (incubation at 37°C in media with 2 mM cold methionine) was required prior to the PBS wash to eliminate free label sequestered in the cells that could not be eliminated by the PBS wash. (Appendix B.6). A minimum one hour chase in media with 2 mM cold methionine was used following each electrical experiment. Six washes of 10 minutes in 1 ml PBS were shown to eliminate the free label in the plug fluid volume.

(4) Other culture parameters—frequency of media changes and volume of media required— were similarly based on previous control studies [72].

### 2.2.1 Chemicals

Dulbecco's Modified Eagles Medium (DMEM), Dulbecco's Phosphate Buffered Saline (PBS), antibiotics (Penicillin and Streptomycin), HEPES, and ascorbate were obtained from Gibco (Grand Island, N.Y.). Type II Collagenase was obtained

from Cappel/Worthington (Malvern, PA). Ultrapure collagenase was obtained from American Biofacturers (Lynbrook, N.Y.). Proline, MEM non-essential amino acids, EDTA, papain and molecular weight markers (MW-SDS-70L, MW-SDS-200), and 2,5-diphenyloxazole (PPO) were obtained from Sigma (St. Louis, MO). NuSerum (NS) was obtained from Collaborative Research (Waltham, MA). Fetal Bovine Serum (FBS) was obtained from HyClone Laboratories Inc. (Logan, Utah). All chemicals used for electrophoresis were electrophoresis grade.  $^{35}\text{S}$ -methionine was obtained from Amersham (typical activity 1000-1500 mCi/mmol).

### **2.2.2 Explant Culture**

#### **Tissue Excision**

Cartilage samples were obtained from the femoropatellar groove of 1-2 week old calves. The joint was opened aseptically, and a cylindrical saw was used to bore a cartilage core down to the bone. Core positions were identified as shown in Fig. 2.1. The core was removed, placed in a sledge microtome and sectioned into 1 or 2 mm thick slices. The first approximately 100  $\mu\text{m}$  was removed to ensure a plane parallel geometry (and eliminate the superficial layer of cells, see Chapter 1). The resulting slices were kept in cold sterile PBS with antibiotics (Penicillin 100 units/ml and Streptomycin 100 $\mu\text{g}$ /ml) until all cores were cut. These were then punched with a dermal punch (Miltex Keyes Dermal Punch, Allmed Surgical Supplies, Arlington, MA) to obtain either two sample plugs, 1/4 inch in diameter and 1 mm thick, or 4 to 5 samples, 3mm in diameter and 2 mm thick.

#### **Explant Maintenance**

Following tissue excision, samples were routinely maintained individually (1/4 inch plugs) or together with samples from a single core (3mm plugs) in 24 well culture dishes in DMEM supplemented with 0.1% NuSerum (DMEM-N), and with antibiotics for the first 2 days. Media was changed every two days. Previous

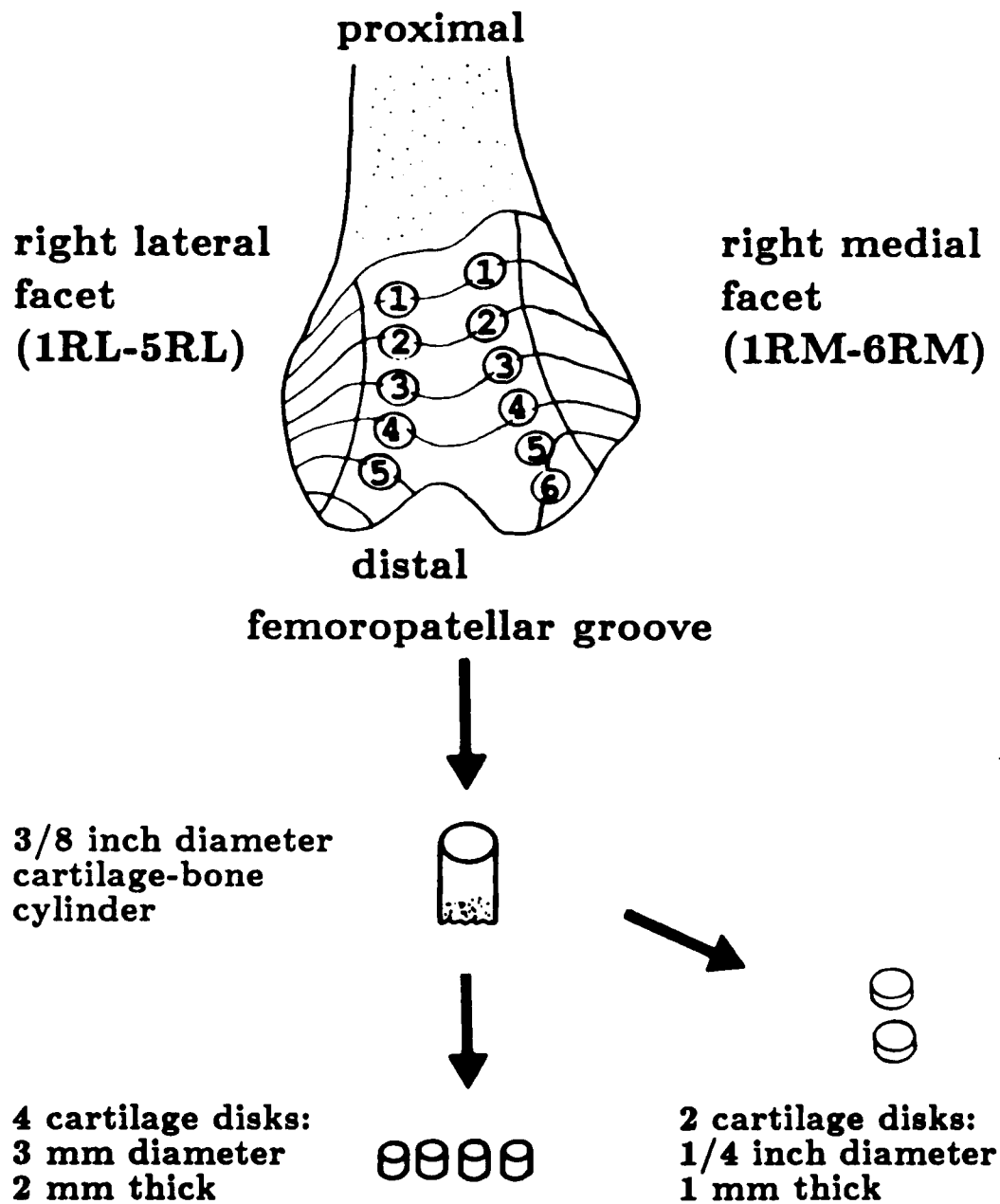


Figure 2.1: Schematic of femoropatellar groove and cartilage explant procedure. Numerical labels designate the core position on the joint surface (RL = right lateral, RM = right medial, LL = left lateral, and LM = left medial facet).

measurement of biosynthesis of glycosaminoglycans and proteins using radiolabeled precursors had confirmed that the specimens obtained using this explant procedure and cultured under different serum conditions are biologically active and maintain their cartilage phenotypic expression (synthesis of type II collagen) [46,89]. To examine how the protein synthetic response to heat shock depended on culture conditions, samples were maintained in DMEM supplemented with 10% FBS, 10 mM HEPES, 0.4 mM proline, 0.1 mM non-essential amino acids, and 20  $\mu$ g/ml ascorbate (DMEM-F), and with antibiotics for the first day. Media was changed every day.

### **2.2.3 Labelling Protocol**

Protein synthesis was assessed by incubating the samples individually in culture dishes with labelling media containing  $^{35}$ S-methionine typically at 5 or 10  $\mu$ Ci/ml, in DMEM-N or DMEM-F without methionine or with 0.1 mM cold methionine added.

Following the chosen incubation period, plugs were either digested in collagenase (see following) or were incubated for 1 hour in media with 2 mM cold methionine (see Appendix B.6). After the cold methionine chase, samples were washed six times in PBS for ten minutes, then patted dry and weighed (wet weight), lyophilized, and reweighed (dry weight) prior to extraction by boiling in SDS-sample buffer.

### **2.2.4 Matching Cartilage Samples**

Cartilage plugs were matched based on the position on the joint surface from which plugs were obtained. Previous control studies of cartilage cultured in 0.1% NS [53,177] had shown that biosynthetic rates varied over the joint surface, but did not depend on depth in the top 2 mm, and did not depend on the joint (right or left). Therefore, in experiments requiring pairs of experimental to control plugs,

experimental and control plugs were chosen from the same joint position. Within a single experiment, the pairs of experimental and control plugs were chosen so that all pairs (paired according to a single position) were obtained from joint positions with similar biosynthetic rates (e.g., the same or adjacent positions on right and left joints).

### **2.2.5 Extraction of SDS-soluble Proteins and SDS-PAGE**

SDS-soluble proteins were extracted either by (a) isolation of cartilage cells from the tissue by collagenase digestion, followed by SDS solubilization of the isolated chondrocytes or by (b) SDS extraction of proteins in the intact tissue.

For extraction of SDS-soluble proteins by collagenase digestion, (using the method of Kubo and Treadwell [111,200]), plugs were first digested in 24 well culture dishes in a 5% CO<sub>2</sub> incubator for 8-10 hours, with intermittent stirring, using 0.1% collagenase (w/v, Worthington Type II) in DMEM. The resulting chondrocyte suspension was twice centrifuged and the cell pellet resuspended in 1 ml Hank's Buffered Salt Solution (HBSS). The chondrocytes were counted using a haemocytometer, spun down again and lysed in gel electrophoresis sample buffer.

For SDS extraction of proteins in the intact tissue, plugs were boiled intact for ten minutes in 100-150  $\mu$ l SDS sample buffer used for gel electrophoresis (2% SDS, 0.0625M Tris, pH 6.8, 5%  $\beta$ -mercaptoethanol, 10% glycerol and 0.05% bromophenol blue) to extract SDS-soluble molecules. The SDS extraction method was adopted after it was shown that the protein bands obtained by gel electrophoresis were comparable to the collagenase digestion method.

Following either method of extraction of SDS soluble molecules, two aliquots of each sample were counted using an LKB Rackbeta 1211 scintillation counter. Residual cartilage plugs (following SDS extraction only) were then either washed three times for 20 minutes in 0.7 ml SDS sample buffer (without  $\beta$ -mercaptoethanol, glycerol, or bromophenol blue), or boiled for 10 minutes in 1.5 ml SDS sample buffer

to remove SDS-soluble molecules remaining in the plug liquid volume. Aliquots from the SDS wash were counted. The total counts in the SDS wash were added to the total counts in each gel sample, to obtain the total counts in SDS-soluble macromolecules for each plug.

The SDS extract was loaded on a 10% polyacrylamide gel, following the method of Laemmli [114], specifically adapted for the Hoefer Sturdier Gel electrophoresis system [97]. Gels were run overnight at constant voltage (50-100 V). Each sample was loaded individually, so that each lane represents a single cartilage plug. A molecular weight marker sample prepared in the same manner was loaded on at least one lane. The protein bands obtained using SDS extraction and the protein bands obtained using collagenase digestion followed by SDS-solubilization of isolated chondrocytes were the same with one exception. An additional band at 140 kD which was identified as procollagen was observed on gels obtained using the SDS extraction protocol (see Appendix B.1).

Labelled proteins were visualized on the gel by autoradiography or fluorography. The gels were stained with Coomassie Blue to visualize the marker proteins and then destained, or were soaked in destain only. For autoradiography, gels were rehydrated in 7% acetic acid and then dried at 80°C for 2-3 hours. Gels were prepared for fluorography following the method of Skinner and Griswold [188]. The gels were soaked for 5 minutes in 10 volumes acetic acid, followed by 1.5 hours in 10 volumes 20% (w/v) PPO in acetic acid, and then 1/2 hour in distilled water. The gels were then dried at 60°C for 2-3 hours. For both autoradiography and fluorography, the gels were exposed to Kodak X-omat film at -70°C.

### **2.2.6 Papain Digest**

Following SDS extraction and wash, residual plugs were papain digested in 500  $\mu$ l of papain solution [80] (2 volumes PBSE (PBS with EDTA), 1 volume distilled water, 0.08% (w/v) cysteine and 0.015 volumes papain) for a minimum of 24 hours at 60°C.

Aliquots of the papain digest were counted.

### **2.2.7 SDS-soluble Fraction and Papain Digest**

Total incorporation was calculated from the sum of the total counts in the SDS extract and washes and in the papain digest. Radiolabel incorporation in the SDS extract for unheated control cartilage specimens was on the order of 10-30% of the total incorporation.

### **2.2.8 Collagenase Digestion Assay for Procollagen**

Sensitivity to digestion by ultrapure collagenase was used to identify the procollagen band on a gel fluorograph. Several plugs were boiled together for 15 minutes in 750  $\mu$ l Tris buffered saline (0.05 M Tris, pH 7.2, 0.15 M NaCl, 0.01% BSA) with 2% SDS. After boiling, a sample of the extract was frozen without any further treatment. To another 50  $\mu$ l of the boiled extract, 0.25  $\mu$ l of 1 M  $\text{CaCl}_2$ , 25 U collagenase, and protease inhibitors (1  $\mu$ M pepstatin, 0.05  $\mu$ M Soybean Trypsin Inhibitor, 1 mM PMSF, and 1 mM Iodoacetimide), were added. This sample was then incubated for 16 hours at 37°C. After 16 hours, 50  $\mu$ l of 2x sample buffer for gel electrophoresis was added to both samples and both were boiled for 10 minutes, and then run on a 10% polyacrylamide gel as usual.

### **2.2.9 Stress Response to Incubation at Elevated Temperatures**

Stress response to incubation at elevated temperatures was assessed qualitatively from gel fluorographs (or autoradiographs) in terms of changes in synthesis of certain specific proteins (the stress or heat shock proteins), and quantitatively in terms of total radiolabel incorporation into the SDS extract and into the papain digest. A severe heat-induced stress (43°C for 6 hours) identified the proteins (stress proteins) whose synthesis was induced by heat.

The stress response was assessed as a function of the severity of the heat



shock: both the temperature and the duration of incubation at elevated temperature were varied. The response was assessed at 37.5, 38.5, 41, 42, 43 and 45°C. At each temperature, the experimental plugs were incubated for a given time at the elevated temperature, followed by incubation at 37°C. The time of incubation at elevated temperature was varied within an experiment, in increments between 0 and the total labelling time, but the total labelling time was held fixed. Matched controls were incubated and labelled at 37°C for the same total labelling time. In each case, culture dishes filled with labelling media were set into the incubator 1/2 hour before the plugs were transferred. Incubator temperature was measured by a thermometer placed adjacent to the dishes. Plugs were incubated at (a) at 37.5 and 38.5 °C for 1-6 hours (1 hour intervals, 1 plug/interval) and labelled for 6 hours. (b) at 41° for 1/2 to 3 hours (in half hour intervals, 1 plug/interval) and labelled for 3 hours; (c) at 42°C for 1/2 to 3 hours (in half hour intervals, 1 plug/interval) and labelled for 3 hours (d) at 43°C for 1,2,3 or 6 hours and labelled for 6 hours and (e) 45°C for 20-70 minutes (in ten minute intervals, 1 plug/interval) and labelled for 3 hours. Table 2.1 summarizes these experiments.

The stress response to heat stress in plugs that had previously been subjected to mild heating, relative to unheated control plugs was compared. Plugs were incubated after excision at either 39 or 37°C (control plugs) for two days prior to incubation at 42°C for 0.5, 1, 1.5, 2, 3, 4, 5 or 6 hours (1 specimen/interval).

#### **2.2.10 Change in Stress Response and Protein Synthesis During Culture**

To assess the stability of protein synthesis during the culture period, especially in the stress response to heat, cartilage plugs were either incubated at 37° or at elevated temperatures (41°C or 43°C for 3 hours, or 45°C for 0.5 hours) and labelled for 3 hours on each day of culture. Table 2.2 summarizes these experiments for reference.

In two experiments, cartilage plugs were labelled with <sup>35</sup>S-methionine at

temperature (°C)	labelling time (hours)	exposure times	day
37.5	6	0,1,2,3,4,5,6 hours	4
38.5	6	0,1,2,3,4,5,6 hours	2
41	3	0,0.5,1,1.5,2,2.5,3 hours	2
42	3	0, 0.5,1, 1.5, 2, 2.5, 3	5
43	6	0,1,2,3,6	7
45	3	0,20,30,40,50,60,70 min	1

Table 2.1: The heat shock experiments done are listed in terms of the temperature of incubation for heat shock, the total labelling time, the range of exposure times and the day after tissue excision on which the experiment was done.

days	temperature (°C)	labelling time (hours)	time at high temperature (hours)
0-5	45	3	1/2
0-7	43	3	3
0,1,5,7,9	41	3	3

Table 2.2: The experiments done to investigate changes in protein synthesis with time in culture, and in the stress response are listed. In each experiment a set of plugs were incubated at 37°C for the total labelling time on each day, and another set was incubated at elevated temperatures. The experiments are listed in terms of the day after tissue explant on which the labelling was done, the incubation temperature used to induce the stress response, the total labelling time, and the incubation time at the elevated temperature.

5 $\mu$ Ci/ml on sequential days of culture. In the first, 36 (1/4 inch diameter) plugs were divided into six groups of 6 and incubated at 37°C. On day 0 (after removal from the joint, and a short time in media at room temperature) and on each subsequent day until day 5, six plugs were labelled with <sup>35</sup>S-methionine (5 $\mu$ Ci/ml) for a total of three hours, three control plugs at 37°C and 3 plugs at 45°C for 1/2 hour, followed by 2.5 hours at 37°C. In the second, a similar protocol was followed over a period of 8 days, but plugs were incubated at 43°C for 3 hours (4 plugs—one 1/4 inch diameter plug and three 3mm diameter plugs—at 43°C, and 4 plugs—two 1/4 inch diameter plugs and two 3 mm diameter plugs—at 37°C on each day). In both experiments, plugs were matched so that plugs with similar rates of synthesis, based on position on the joint surface [177,53] (Appendix B.8), were labelled over the test period (6 or 8 days) for a given treatment (heat shock or control). However, heat shocked plugs were not matched to control plugs.

A final experiment was done to investigate how alterations in synthesis with time in culture might depend on the specific culture media used, and to investigate alterations over a longer culture period. Cartilage plugs, 3 mm in diameter, were maintained either in DMEM-N or in DMEM-F, as described in Section 2.1.2 in Explant Maintenance. Plugs maintained in DMEM-N were labelled in DMEM-N with 0.1 mM cold methionine and 8.5  $\mu$ Ci/ml <sup>35</sup>S-methionine. Plugs maintained in DMEM-F were labelled in DMEM-F with 0.1 mM cold methionine and 8.5  $\mu$ Ci/ml <sup>35</sup>S-methionine. Plugs were incubated and labelled for 3 hours on days 0,1,5,7, and 9 at 37°C or at 41°C. The plugs were matched such that one plug from each core position was labelled on each day, and so that plugs incubated at 41°C were matched to control plugs by core position on the joint surface (matching right to left joint).

## 2.3 Results

### 2.3.1 Heat Induced Alterations in Protein Synthesis

#### Stress Protein Synthesis

Increased synthesis of several distinct proteins was observed (assessed qualitatively from the gel fluorograph) in samples incubated at 43°C for 6 hours, under severe heat shock conditions (Fig. 2.2).

These included proteins of apparent molecular weight 97, 90, 78, 70, 62, 57, 49 and 41 kD (as determined by measurement relative to front on the gel, see Appendix A). Some protein bands had no visible counterpart in the fluorograph lanes corresponding to unheated controls (the 97, 70 and 41 kD proteins). The intensity of the others (the 90, 78, 62, 57, and 49 kD proteins) was enhanced on fluorographs of heat shocked relative to unheated controls. In this and in all other heat shock experiments the most prominent was the 70 kD stress protein (here designated SP70), which had previously been identified as a major stress or heat shock protein in chondrocyte cell culture [132,131,111]. Induction of the 97 and 90 kD proteins accompanied induction of SP70 at all temperatures for which SP70 was induced. Induction of the 78 kD protein became apparent only after longer exposures to heat. The other proteins did not appear to be induced under less severe heat shock conditions (lower temperatures and shorter times). A protein of approximately 110 kD was induced only in plugs that had been incubated for over 1/2 hour at 45°C.

SP70 was synthesized in cartilage plugs that had been incubated at 41°C (on day 2 of culture), 42°C (on day 5 of culture), 43°C (on day 7 of culture) and 45°C (on day 1 of culture), but not at 37.5 or 38.5°C. The intensity of SP70 synthesis increased with the length of incubation time at elevated temperature, for lower temperatures (e.g., 41°C (Fig. 2.3) and 42°C), but not at 43°C or 45°C (for the time increments investigated), as assessed qualitatively by examination of the

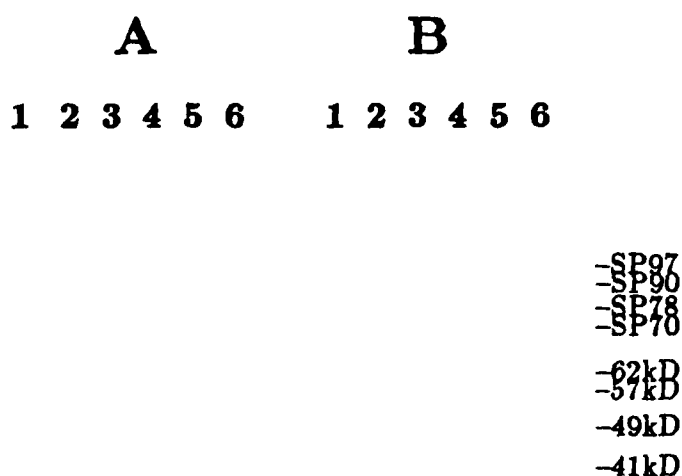


Figure 2.2: (A) Control plugs incubated and labelled at 37°C for 6 hours (fluorograph).

(B) Heat-shocked plugs incubated and labelled at 43°C for 6 hours. In both (A) and (B) each lane corresponds to a single plug incubated in labelling media on day 3 with concentrations of added cold methionine of (1) 0 mM, (2) 0.001 mM, (3) 0.01 mM, (4) 0.1 mM, (5) 1.0 mM and (6) 2.0 mM. Protein bands that are more intense for the heat-shocked specimens relative to their matched controls are labelled according to molecular weight. Those designated SP were seen consistently following incubation at the elevated temperatures. Induction of the lower molecular weight proteins was not apparent under less severe heat shock.

Plugs were prepared for electrophoresis using the SDS extraction method. (see Appendix B for a discussion of incorporation in different concentrations of cold methionine.)

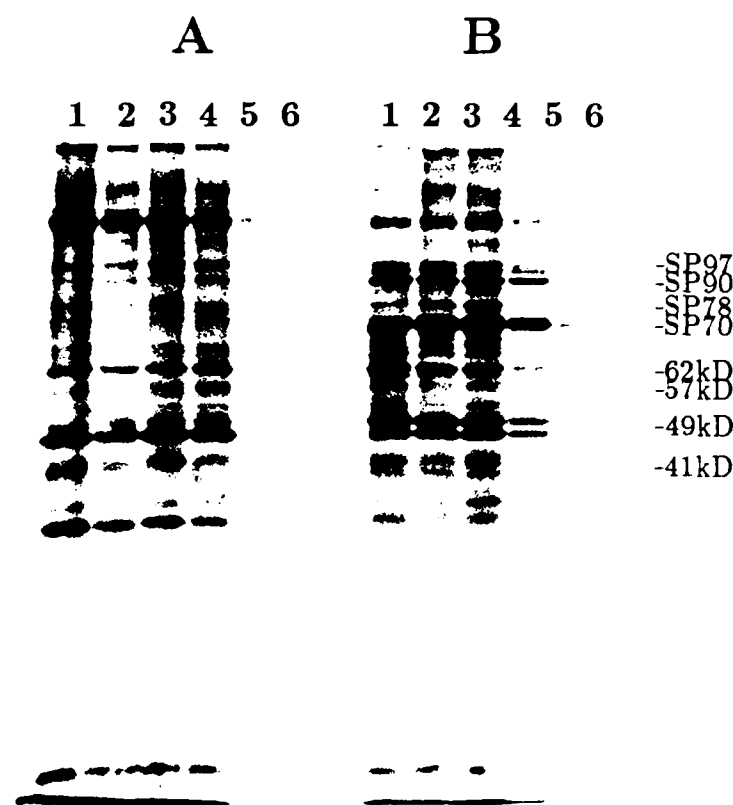


Figure 2.2: (A) Control plugs incubated and labelled at 37°C for 6 hours (fluorograph).

(B) Heat-shocked plugs incubated and labelled at 43°C for 6 hours. In both (A) and (B) each lane corresponds to a single plug incubated in labelling media on day 3 with concentrations of added cold methionine of (1) 0 mM, (2) 0.001 mM, (3) 0.01 mM, (4) 0.1 mM, (5) 1.0 mM and (6) 2.0 mM. Protein bands that are more intense for the heat-shocked specimens relative to their matched controls are labelled according to molecular weight. Those designated SP were seen consistently following incubation at the elevated temperatures. Induction of the lower molecular weight proteins was not apparent under less severe heat shock.

Plugs were prepared for electrophoresis using the SDS extraction method. (see Appendix B for a discussion of incorporation in different concentrations of cold methionine.)

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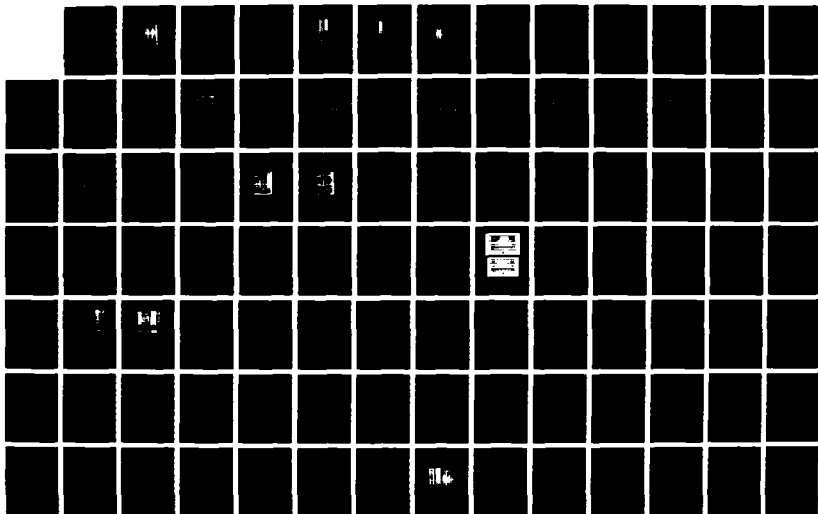
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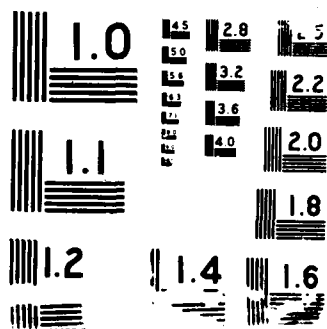
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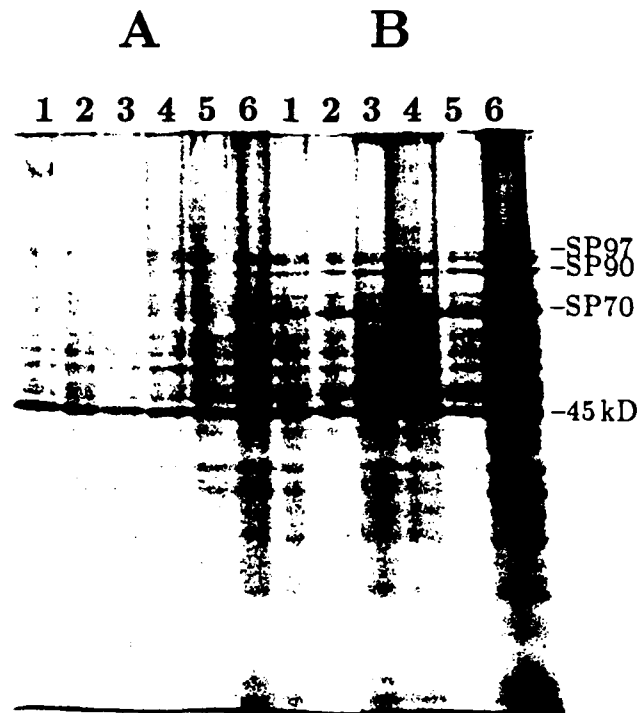


Figure 2.3: Cartilage plugs incubated at 41°C for 1/2 to 3 hours and their matched controls incubated at 37°C (autoradiograph). Each lane corresponds to a single plug prepared using the collagenase digestion method.

(A) Control plugs incubated and labelled for 3 hours at 37°C.

(B) Plugs incubated and labelled at 41°C for 1/2 hour (lane 1), 1 hour (2), 1.5 hours (3), 2 hours (4), 2.5 hours (5) and 3 hours (6). Plugs were then returned to 37°C for the remainder of the 3 hour labelling period. Three stress protein bands are labelled by molecular weight in kD (SP90 and SP70).

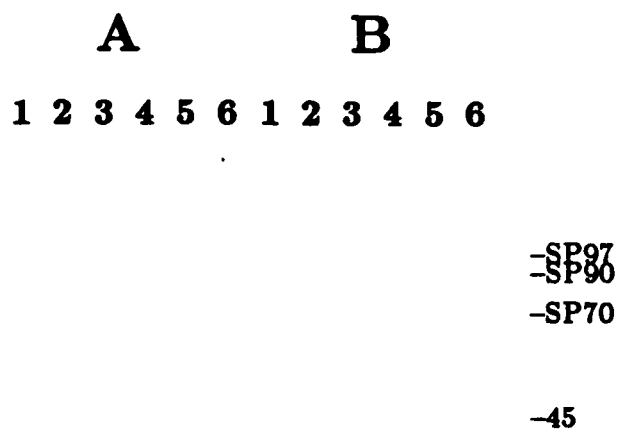


Figure 2.3: Cartilage plugs incubated at 41°C for 1/2 to 3 hours and their matched controls incubated at 37°C (autoradiograph). Each lane corresponds to a single plug prepared using the collagenase digestion method.

(A) Control plugs incubated and labelled for 3 hours at 37°C.

(B) Plugs incubated and labelled at 41°C for 1/2 hour (lane 1), 1 hour (2), 1.5 hours (3), 2 hours (4), 2.5 hours (5) and 3 hours (6). Plugs were then returned to 37°C for the remainder of the 3 hour labelling period. Three stress protein bands are labelled by molecular weight in kD (SP90 and SP70).

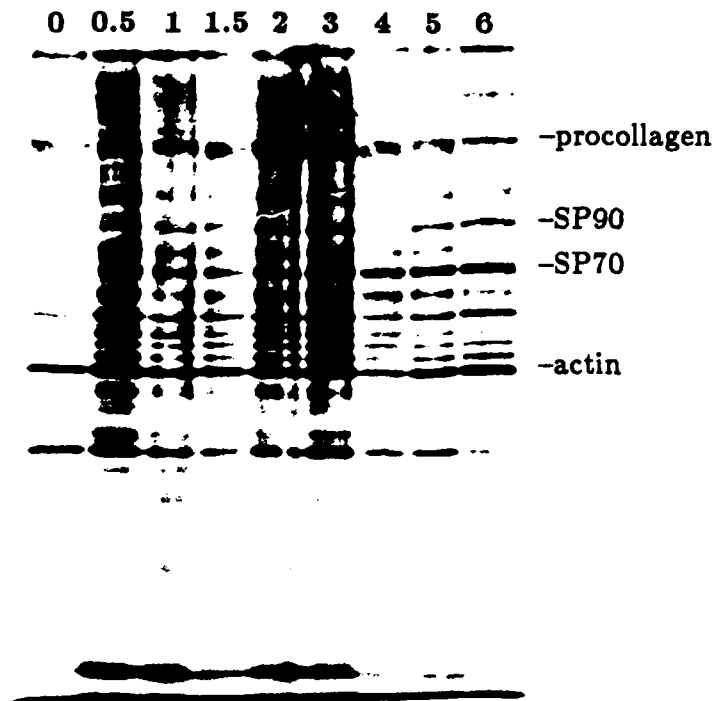
gel autoradiographs. Qualitative comparison of fluorographs for each temperature suggested that SP70 synthesis increased with temperature between 41 and 43°C.

At 41°C, SP70 intensity increased with incubation time at 41°C between 0-3 hours. At 42°C, induction appeared to saturate at 1.5 hours (data not shown), but this may have been due to saturation of film sensitivity using fluorography. At 42°C (Fig. 2.4 (A)), following incubation at 37°C for two days, the intensity of the SP70 band increased for incubation times between 0 and 6 hours. For plugs that had previously been incubated at 39°C, the intensity of the band appeared to increase from 0 to 3 hours, but for longer incubations there was no further apparent increase in intensity

(Fig. 2.4). At 43°C (0-6 hour on day 7), there was no observed change in the intensity of SP70 with incubation time at 43°C, for incubation times of greater than 1 hour (Fig. 2.5). Similarly at 45°C, no change in the intensity of SP70 synthesis was observed between different lengths of incubation time at 45°C. No SP70 synthesis was observed at 37.5°C or 38.5°C for incubations of up to 6 hours, nor in plugs labelled while incubated at 37°C for 6 hours following a 2 day incubation at 38.5-39°C. However, plugs incubated at 38.5-39°C for two days did not exhibit as strong a stress response to subsequent incubation at 43°C, (in terms of the intensity of the SP70 band induced), compared to plugs that had been pre-incubated at 37°C (Fig. 2.4).

#### **Decrease in Collagen Synthesis**

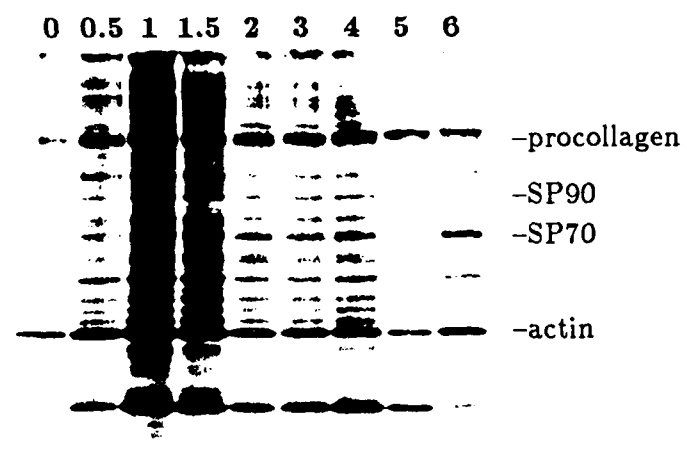
In two experiments done within the first 4 days of culture, SP70 synthesis was also accompanied by a decrease in the intensity of the protein band at 140 kD. This band was identified as procollagen by its specific sensitivity to collagenase digestion. Following digestion of one half of the gel sample (see Methods), the 140 kD protein band was eliminated in the lane corresponding to the digested half of the sample, while no other protein bands showed any apparent change in intensity, relative to the lane corresponding to the undigested half sample.



(A)

Figure 2.4: Gel fluorographs of samples labelled at 43°C for 0-6 hours, following preincubation either at 37 or 39°C for two days. The lane number designates the duration of incubation at 43°C in hours. Samples were prepared by SDS extraction method.

(A) Samples preincubated for 2 days at 37°C



(B)

Figure 2.4: (B) Samples preincubated for 2 days at 39°C

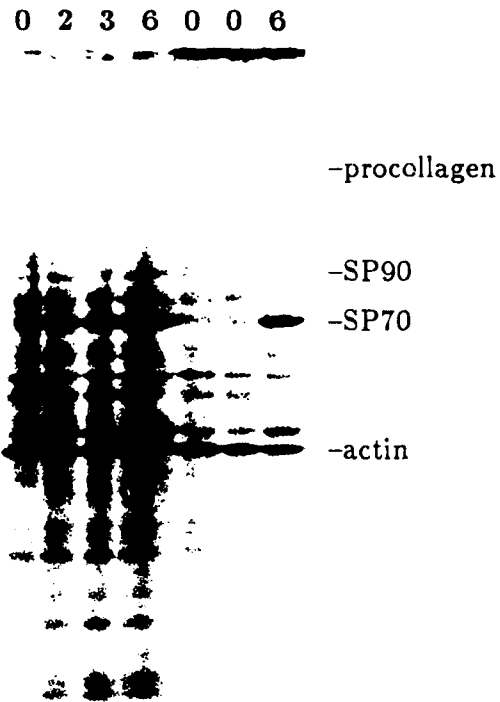


Figure 2.5: Gel fluorographs of samples labelled at 43°C for 0-6 hours. The lane number designates the duration of incubation at 43°C in hours.

Samples were prepared by the SDS extraction method.

0 2 3 6 0 0 6

-procollagen

-SP90

-SP70

-actin

Figure 2.5: Gel fluorographs of samples labelled at 43°C for 0-6 hours. The lane number designates the duration of incubation at 43°C in hours.

Samples were prepared by the SDS extraction method.

There is a clear decrease in the intensity of the procollagen band corresponding to samples heated at 43°C for 6 hours relative to the procollagen band for controls incubated at 37°C (Fig. 2.2). It is not clear from examination of the gels how this decrease depended on the duration of incubation at the elevated temperature in the experiment at 43°C for 0 to 6 hours on day 2 with prior incubation at either 37 or 39°C (Fig. 2.4). However, the decrease was greater for the cartilage plugs incubated at 37°C prior to incubation at 43°C. In two early experiments (41° for 0 to 3 hours, on day 2 and 45°C for 0 to 70 min on day 1), the samples were processed by collagenase digestion, followed by SDS solubilization, so that the procollagen band did not appear. The decrease in intensity of the procollagen band during heat shock was not observed in experiments done later in the culture period.

The decrease in the intensity of the procollagen band was similarly observed when SP70 was induced by electrode reaction products (in electrical stimulation experiments, Chapter 3). In this case, the intensity of the procollagen band was quantitated by densitometry of the gel autoradiograph. The decrease in the ratio of the procollagen band intensity to the actin band intensity for experimental samples relative to their matched controls was significant ( $p < 0.02$ , experimental/control = 0.52).

### Total Protein Synthesis

Heat induced stress altered total radiolabel incorporation both into the SDS extract and in the papain digest. In 0.1%NS, synthesis of SDS-soluble proteins increased slightly in heat shocked relative to control samples, for cartilage samples that had previously been maintained in culture for a long enough time (greater than 4 days, see next section). Incorporation into the papain digest increased for short times at high temperature, and then decreased.

An increase in incorporation in the SDS extract of approximately 30-50% was maintained from 1-6 hours of incubation at 43°C (Fig. 2.6).



# Incubation for 0-6 hours at 43 degrees

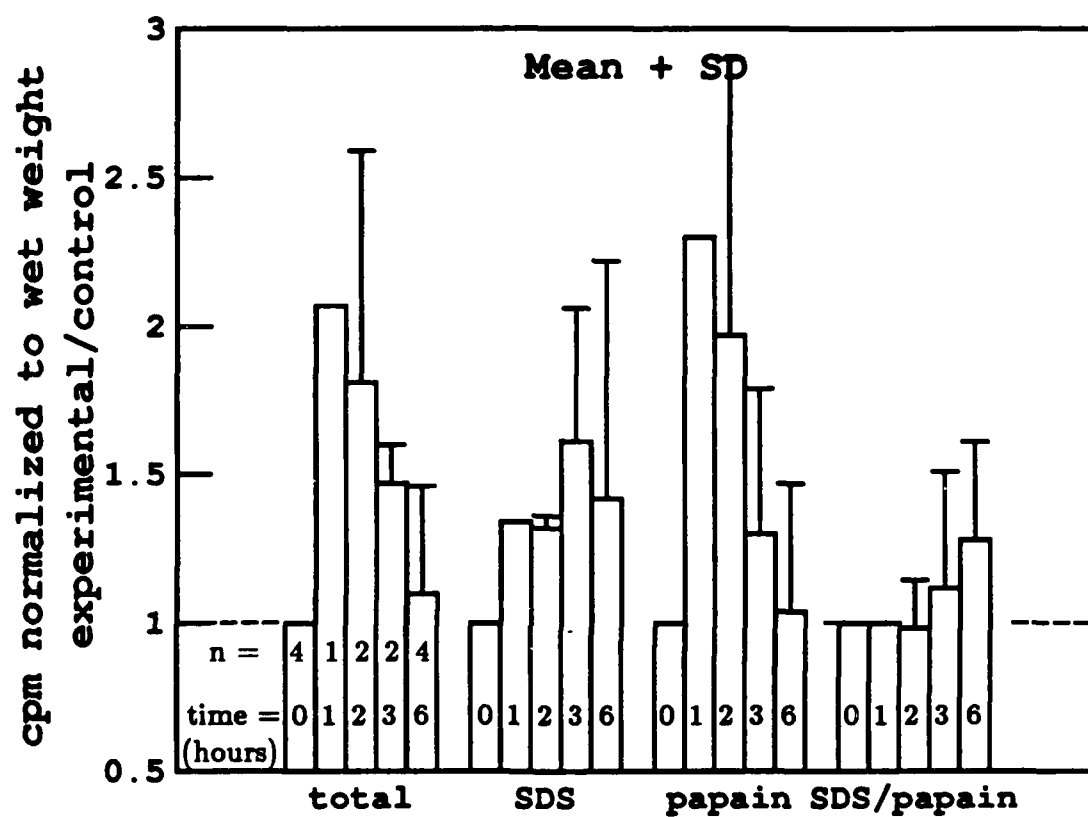


Figure 2.6: Radioactivity in each fraction as a function of incubation time at 43°C is plotted as the ratio of incorporation in heated plugs to unheated controls. The data corresponds to the gel shown in Fig. 2.5.

In experiments in which the specimens were processed by collagenase digestion followed by SDS solubilization, only SDS soluble counts were obtained (43 for 0 to 3 hours, and 45°C for 0 to 70 min). The SDS-soluble counts in both experiments showed no significant difference between heat shocked and control samples (Fig. 2.7). However, both experiments were done early in the culture period (day 1 and day 2) and other studies (section following) demonstrated that differences in synthetic behavior between heated and unheated samples were smaller during the first few days compared to differences later in culture.

In 0.1%NS, radiolabel incorporation in the papain digest also appeared to increase with short heat shock, but then to decrease for longer heat shock. In plugs heated at 42°C for 1/2 or 1 hour, the radioactivity in the papain digests of heated plugs was greater than twice that of matched controls (Fig. 2.8). Incorporation decreased monotonically with duration of heat shock. Between 2-3 hours at 42°C, radioactivity in heated samples was approximately equal to matched controls. Similarly, at 43°C (0-6 hours, on day 7) incorporation in the papain digest first increased and then decreased (Fig. 2.6) to approximately the control level in 6 hours. In experiments assessing changes over the culture period (41 and 43°C for 3 hours), radioactivity in the papain digest of heat shocked plugs decreased relative to controls. No change in the radioactivity in the papain digest was induced by heating at temperatures which did not induce SP70 synthesis (37.5 and 38.5°C for 1-6 hours).

Total incorporation, of which incorporation into the papain digest is a large part, like incorporation into the papain digest, increases for short times and decreases for long times at elevated temperature (see Fig. 2.6 and next section).

The ratio of the counts in the SDS-soluble fraction relative to the papain digest increased (e.g., at 43°C for 3 hours), for long exposures to elevated temperatures (> a few hours). This follows from an increase in incorporation in the SDS extract combined with a decrease or little change in incorporation into the papain

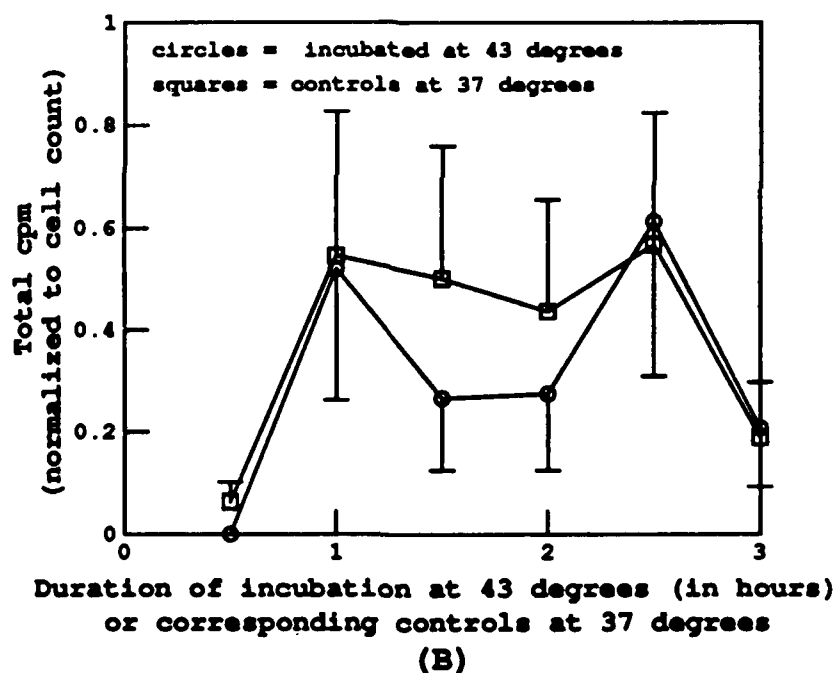
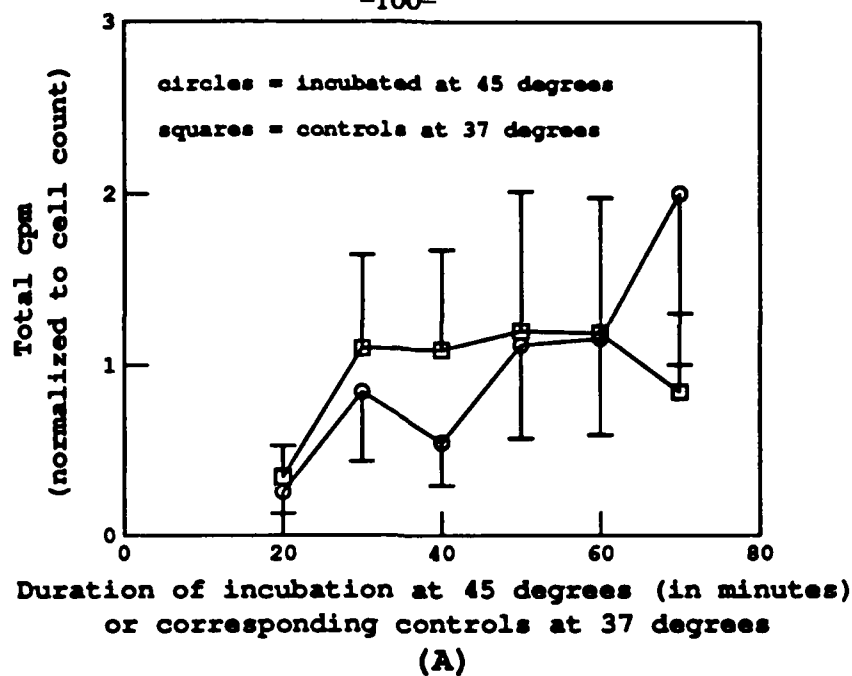


Figure 2.7: SDS-soluble counts normalized to cell count are plotted as a function of the duration of the heat shock. Each point represents the single plug corresponding to a lane on a gel. The bars represent an estimate of the error, based on cell number and volume accuracy.

- (A) plugs incubated at 45°C for 0-70 minutes, and corresponding controls.  
 (B) plugs incubated at 43°C for 0-3 hours (Fig. 2.3), and corresponding controls.

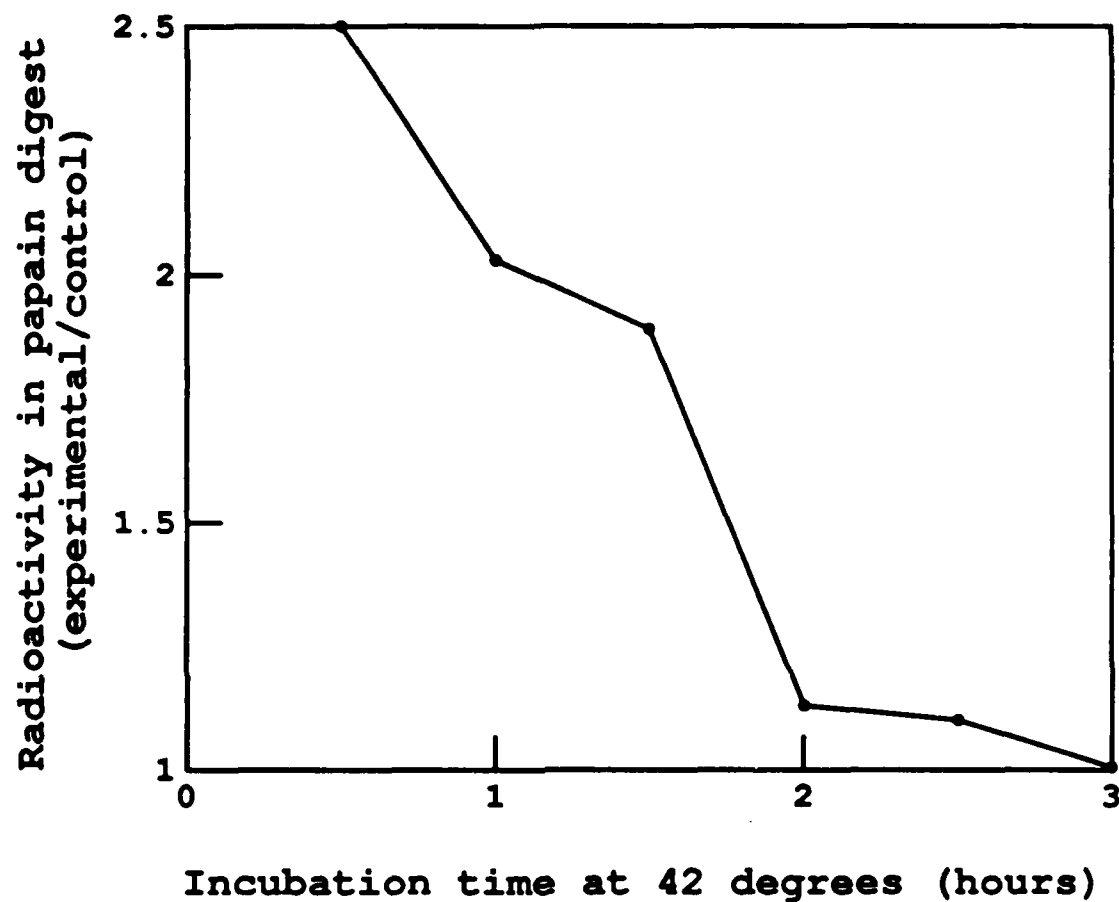


Figure 2.8: Radioactivity in the papain digest as a function of the incubation time at 42°C, is plotted as the ratio of incorporation in heated to control plugs. Each point corresponds to a single experimental and control pair for gel electrophoresis.

digest for long exposures at high temperatures. The change in the ratio in response to heat shock varied with day after tissue excision (see next section).

### **2.3.2 Change in Stress Response and Protein Synthesis With Time in Culture**

#### **Stress Protein Synthesis**

In 0.1%NS, the SP70 band was visible in lanes derived from plugs incubated at 37°C when labelled immediately after tissue excision (day 0) and on days 1,2 and 4 after excision, in the first experiment in which plugs were labelled on each day during the culture period. In the same experiment, it appeared that SP70 synthesis by plugs incubated at 45°C increased between day 0 and day 3 after excision, but this was not certain, due to uneven lane loading (Fig. 2.9). Qualitatively, no further change in SP70 synthesis in heated specimens occurred after 3 days. In the two other experiments, however, SP70 was not visible on gels derived from samples incubated at 37°C on any day after tissue excision. SP70 synthesis by plugs incubated at 43°C was qualitatively the same on all days in culture. (Fig. 2.10). SP70 synthesis by plugs incubated at 41°C appeared to increase between day 0 and day 1 (Fig. 2.20). In the same experiment, SP70 also increased between day 0 and day 1 in plugs that had been maintained in 10% FBS. However, on all days, the intensity of the SP70 band relative to the intensity of normal proteins in the same lanes, qualitatively appeared to be less in lanes corresponding to plugs maintained in 10%FBS than in lanes corresponding to plugs maintained in 0.1%NS, and labelled on the same day.

#### **Total Protein Synthesis**

In 0.1%NS, total counts in the SDS-soluble protein fraction first increased on day 1 (for heat shocked plugs) or remained the same (for controls), and then decreased and reached a steady state on days 2-3 (Fig. 2.11 and 2.15) in culture. Incorporation in the papain digest, usually increased slightly between day 0 and day 1 (with one

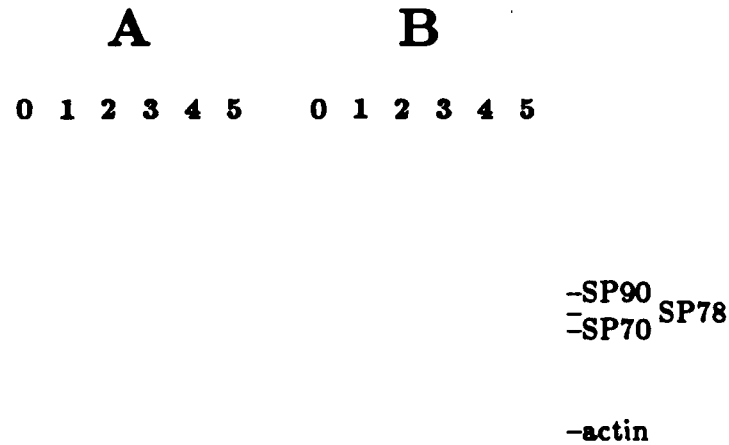


Figure 2.9: Cartilage plugs labelled on days 0-5 after tissue excision (autoradiograph). In both (A) and (B), lane numbers correspond to a single plug labelled on that number day (i.e. day 0 in lane 0). Plugs were prepared using the collagenase digestion method.

(A) Plugs incubated at 37°C and labelled for 3 hours. (B) Plugs incubated at 45°C for 1/2 hour, followed by 2.5 hours at 37°C (while labelled for 3 hours)

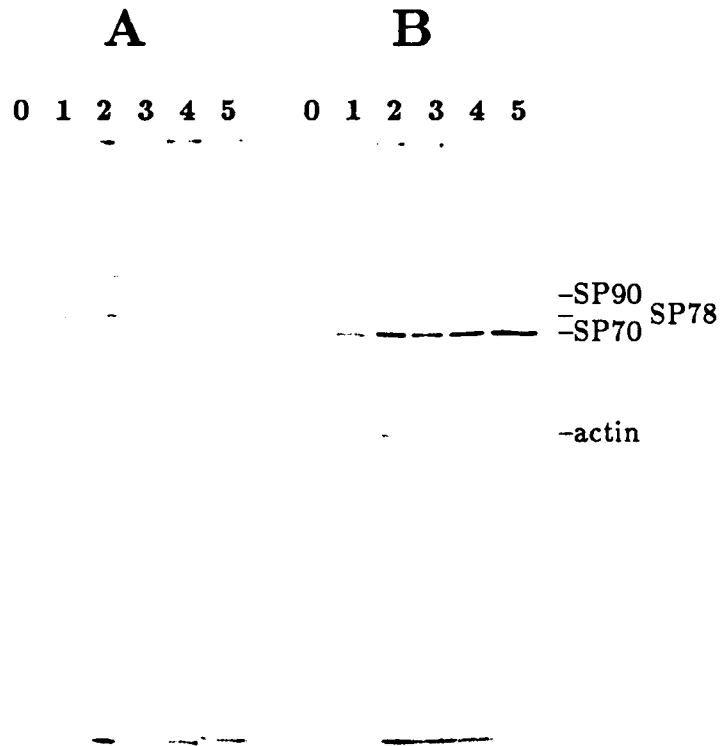


Figure 2.9: Cartilage plugs labelled on days 0-5 after tissue excision (autoradiograph). In both (A) and (B), lane numbers correspond to a single plug labelled on that number day (i.e. day 0 in lane 0). Plugs were prepared using the collagenase digestion method.

(A) Plugs incubated at 37°C and labelled for 3 hours. (B) Plugs incubated at 45°C for 1/2 hour, followed by 2.5 hours at 37°C (while labelled for 3 hours)

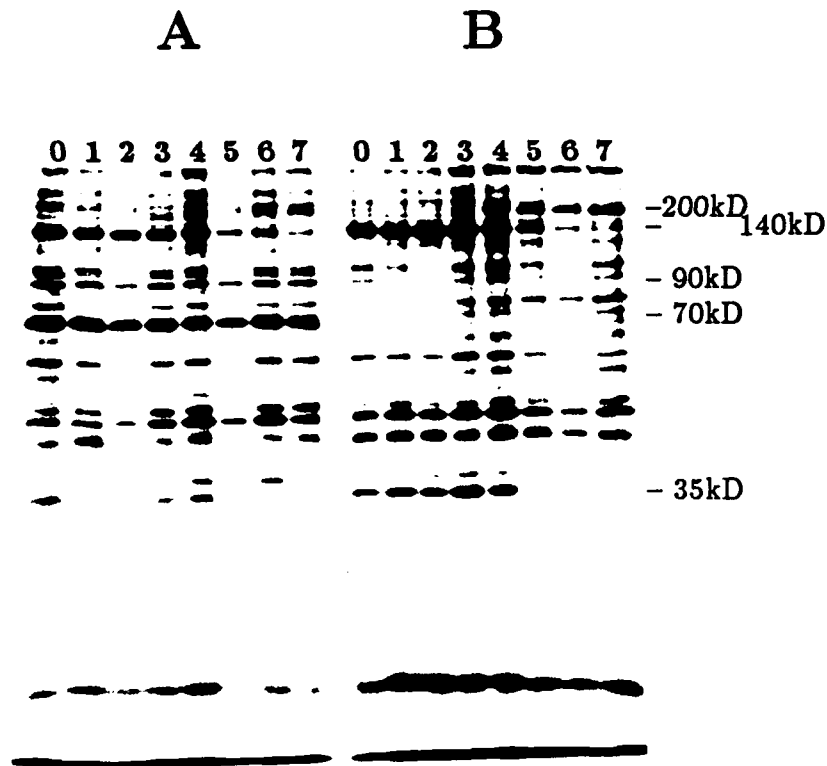


Figure 2.10: Cartilage plugs labelled on days 0-7 after tissue excision (fluorograph). In both (A) and (B), lane numbers correspond to a single plug labelled on that number day (i.e. day 0 in lane 0). Plugs were prepared using the SDS extraction method.

(A) Plugs incubated at 43°C and labelled for 3 hours

(B) Plugs incubated at 37°C and labelled for 3 hours.

The SP70 and SP90 bands are labelled. In addition, protein bands whose intensity decreased on days 5-7 (140 and 35 kD), and those whose intensity increased (200 and 78 kD) are labelled according to molecular weight.



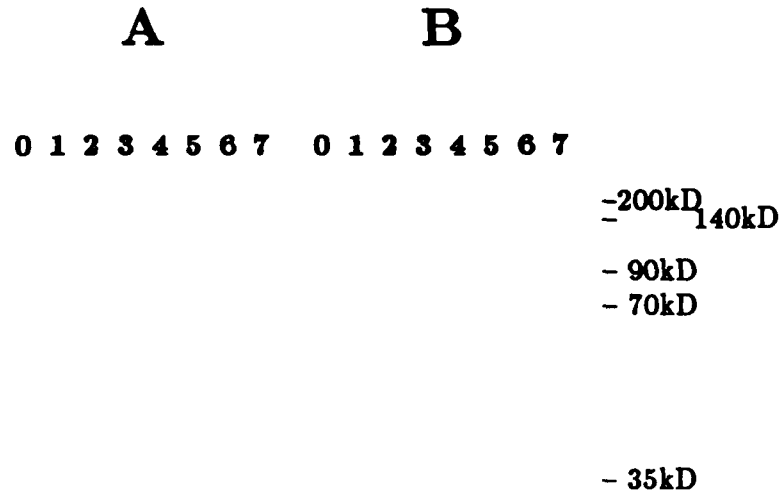
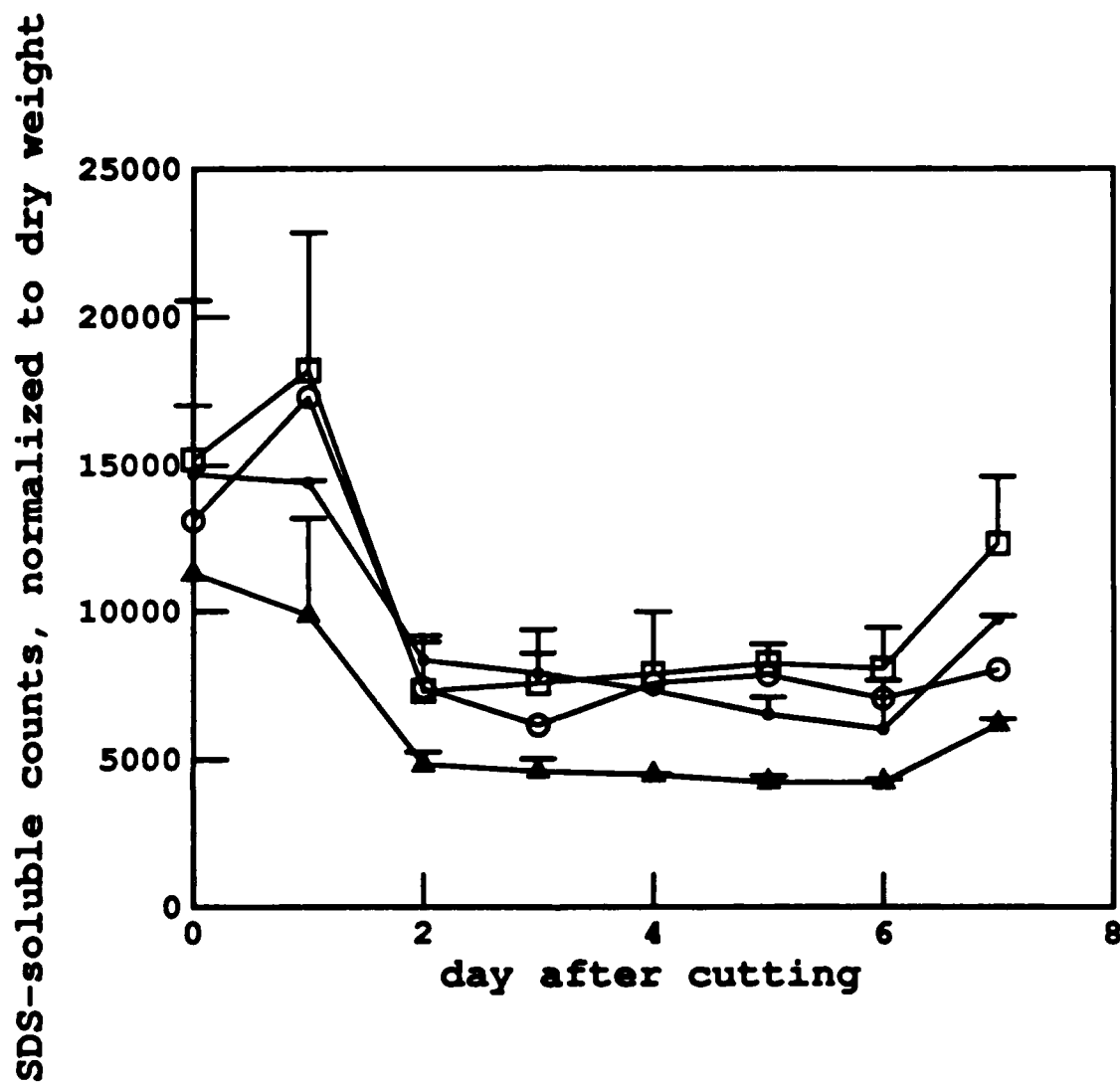


Figure 2.10: Cartilage plugs labelled on days 0-7 after tissue excision (fluorograph). In both (A) and (B), lane numbers correspond to a single plug labelled on that number day (i.e. day 0 in lane 0). Plugs were prepared using the SDS extraction method.

(A) Plugs incubated at 43°C and labelled for 3 hours

(B) Plugs incubated at 37°C and labelled for 3 hours.

The SP70 and SP90 bands are labelled. In addition, protein bands whose intensity decreased on days 5-7 (140 and 35 kD), and those whose intensity increased (200 and 78 kD) are labelled according to molecular weight.



circles= 43 degrees, 1/4 inch plugs  
squares = 43 degrees, 3mm plugs  
triangles= 37 degrees, 1/4 inch plugs  
dots = 37 degrees, 3mm plugs

Figure 2.11: Counts in the SDS extract, normalized to wet weight, are plotted as a function of days after tissue excision.

exception), and then decreased to an apparent steady state, or very slow decline. (Fig. 2.12 and 2.16). Since the radioactive counts in the papain digest is a large fraction of the total (SDS-soluble and papain digest), the total counts varied similarly (Fig. 2.13 and 2.17). The ratio of counts in the SDS-soluble fraction to the counts in the papain digest decreased to day 3, and then increased (Fig. 2.14 and 2.18) to day 7. In the experiment in which plugs were labelled up to 9 days, the ratio appeared to reach a steady state after day 5, between day 7 and 9. The increase in the ratio with day of culture after 4 days was especially marked in samples incubated at elevated temperatures.

In culture using 10%FBS, in contrast to culture using 0.1%NS, incorporation in the SDS extract increased monotonically from day 0 to day 9 in control plugs, and increased up to day 7 in heat shocked plugs (Fig. 2.15). Incorporation in the papain digest, and similarly, total incorporation decreased from day 0 - 1 but then was maintained at approximately the same level (controls) or a slightly higher level (heat shocked plugs) throughout the culture period (Fig. 2.16). Incorporation in both the SDS extract and the papain digest were greater in plugs incubated in 10%FBS than in plugs incubated in 0.1%NS between days 5-9. The difference is significant on day 9 ( $p < .01$  for both fractions). The change with day of culture in the ratio of incorporation in the SDS extract relative to the papain digest is similar in 10%FBS and 0.1%NS (no significant difference on any day). However, the increase in the ratio between day 5-9 was slightly less in 10% FBS, and in 10%FBS, the ratio continued to increase to day 9.

In general, the difference between heated and control plugs in total radioactivity in SDS-soluble or papain digest fractions was variable over the first several days of culture, and became more consistent after about 4 days. After 4 days, in 0.1%NS, incorporation in the SDS extract increased slightly in heated plugs relative to controls. Incorporation in the papain digest decreased slightly, so that the ratio of SDS/papain incorporation increased. After 4 days in 10%FBS, incorporation in

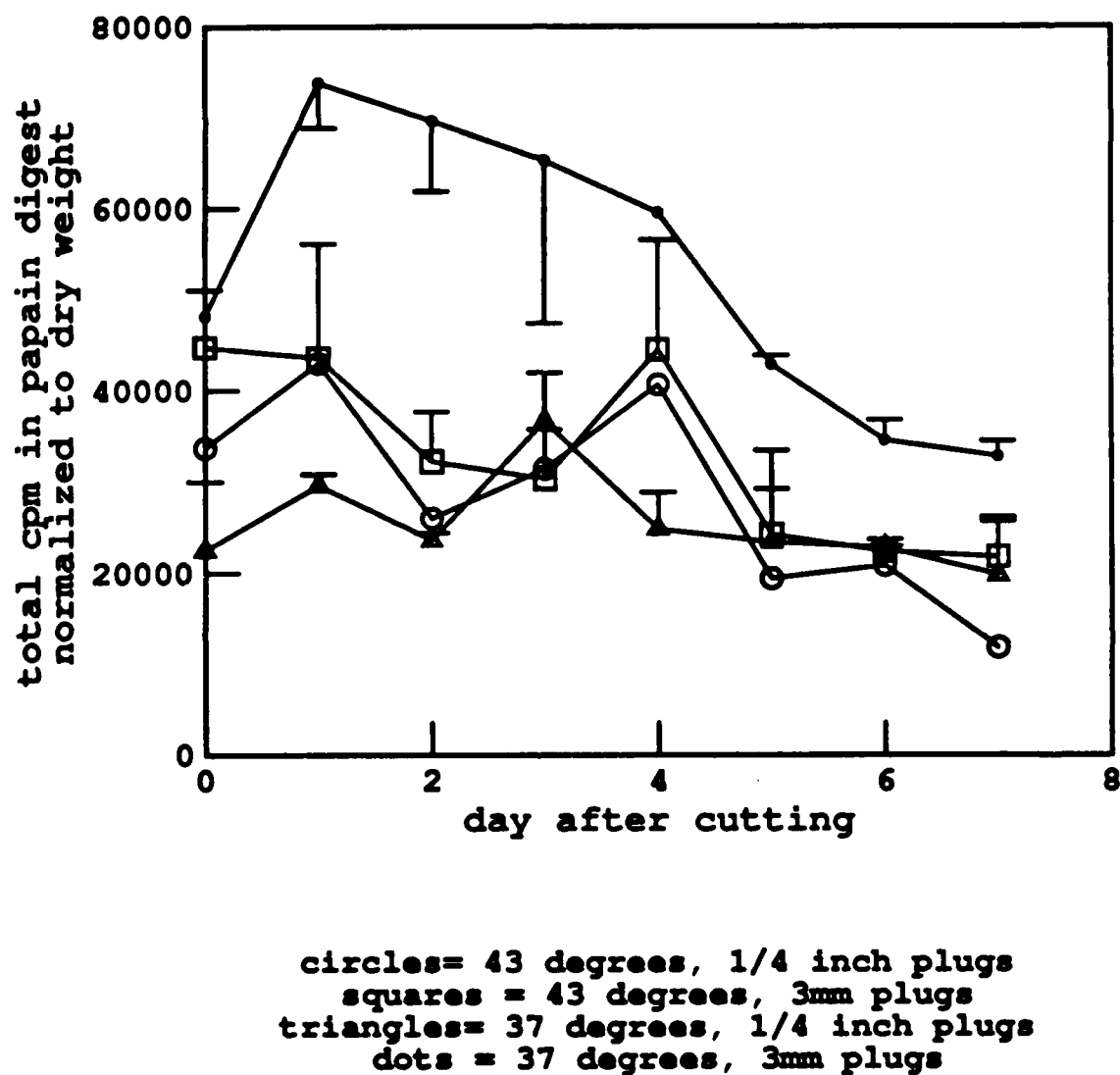
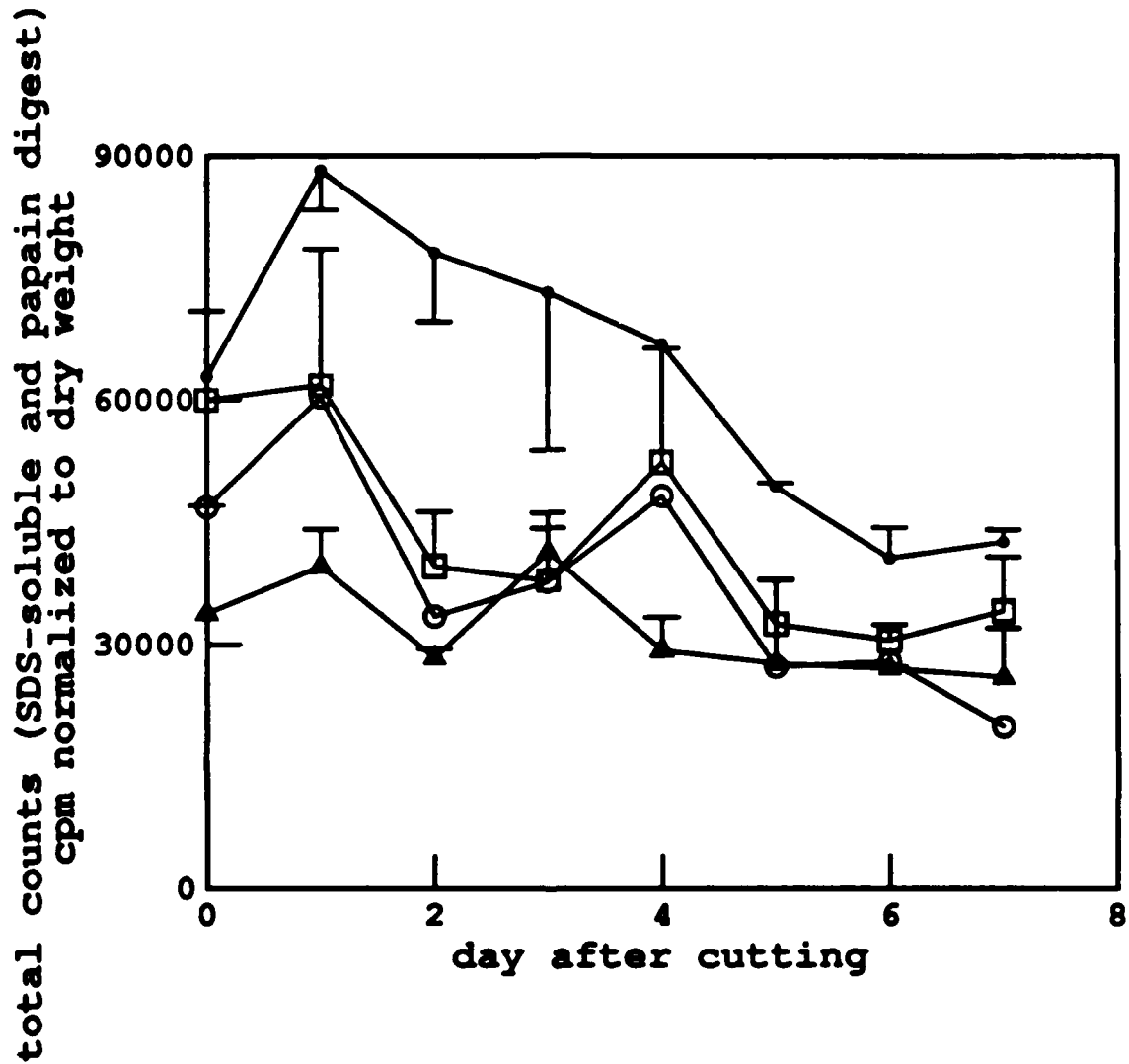
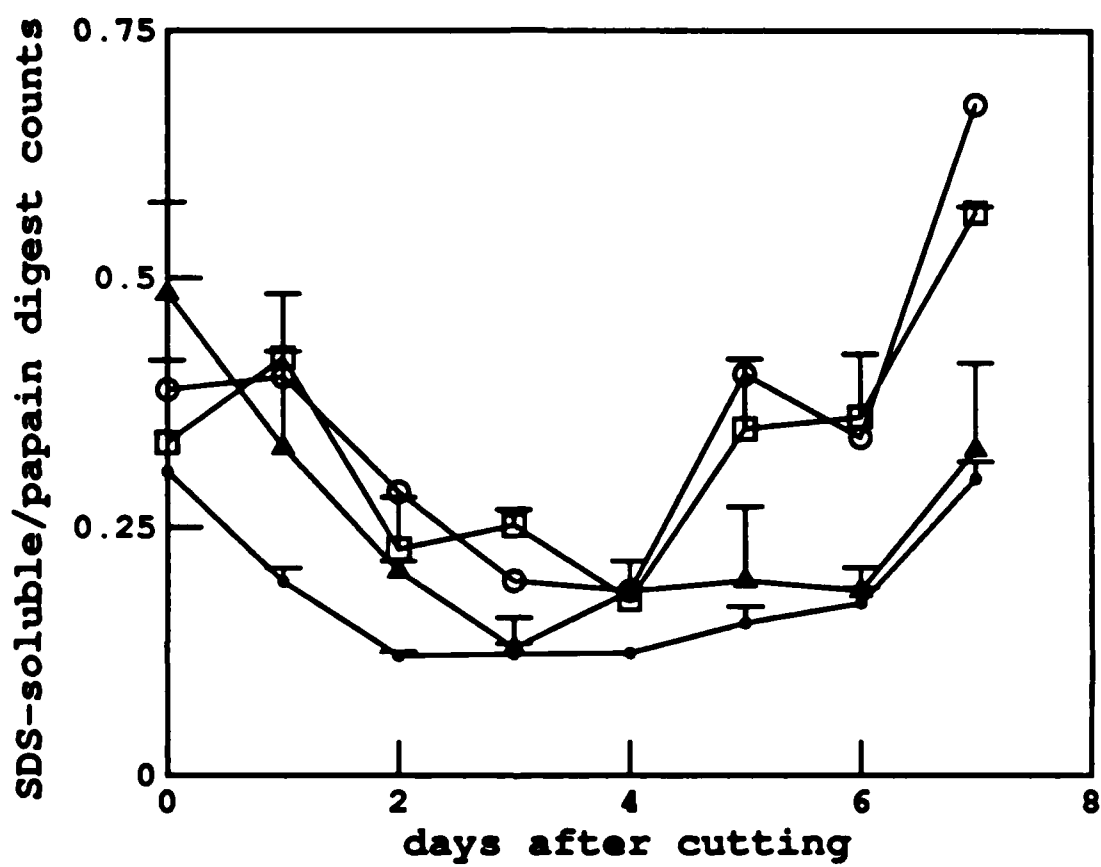


Figure 2.12: Total counts in the papain digest, normalized to wet weight, are plotted as a function of days after tissue excision.



circles= 43 degrees, 1/4 inch plugs  
squares = 43 degrees, 3mm plugs  
triangles= 37 degrees, 1/4 inch plugs  
dots = 37 degrees, 3mm plugs

Figure 2.13: Total counts in the SDS-soluble and papain digest extracts, normalized to wet weight, are plotted as a function of days after tissue excision.



circles = 43 degrees, 1.4 inch plugs  
squares = 43 degrees, 3mm plugs  
triangles = 37 degrees, 1/4 inch plugs  
dots = 37 degrees, 3mm plugs

Figure 2.14: The ratio of counts in the SDS-soluble fraction to the counts in the papain digest are plotted as a function of days after tissue excision.

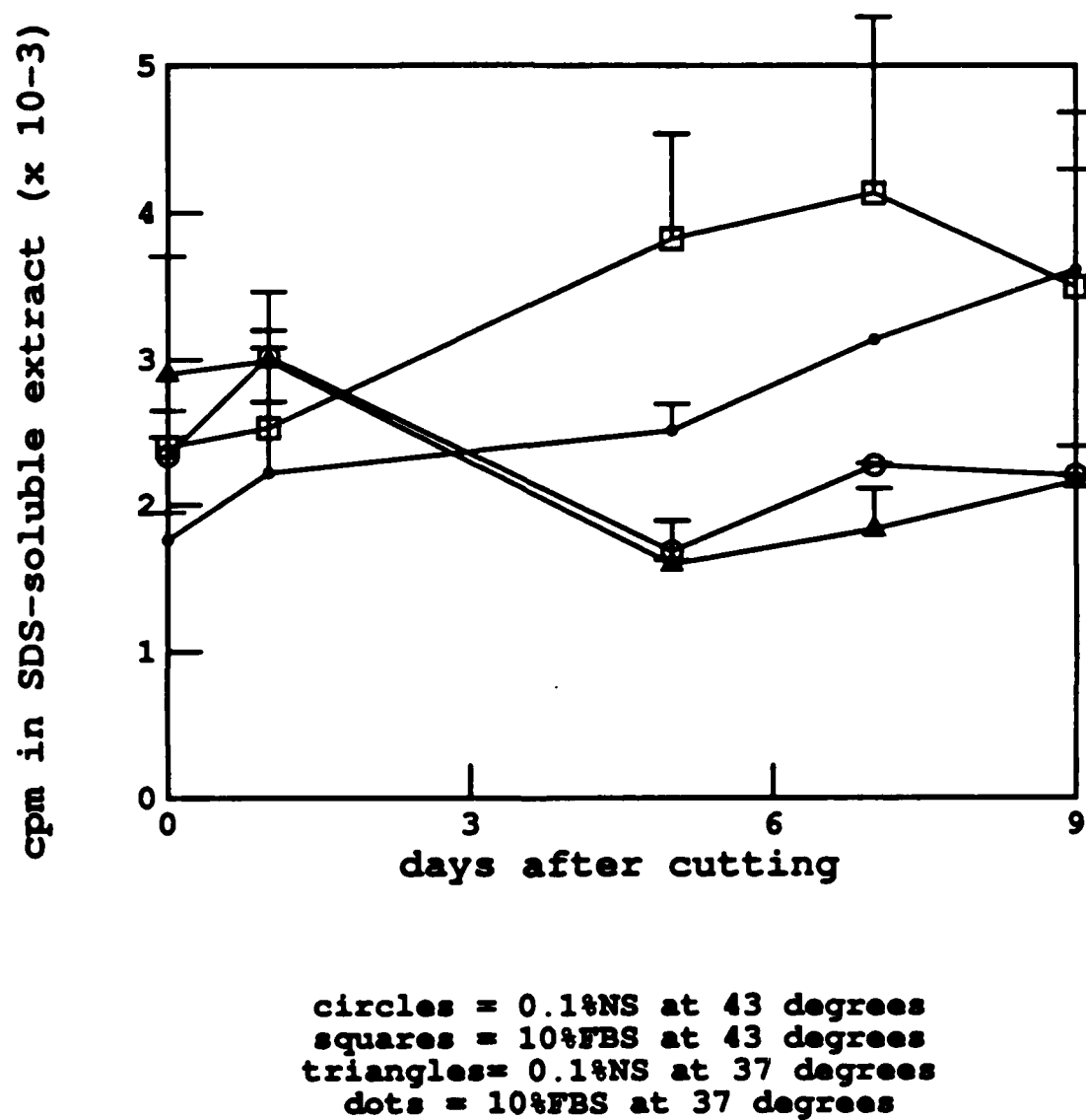
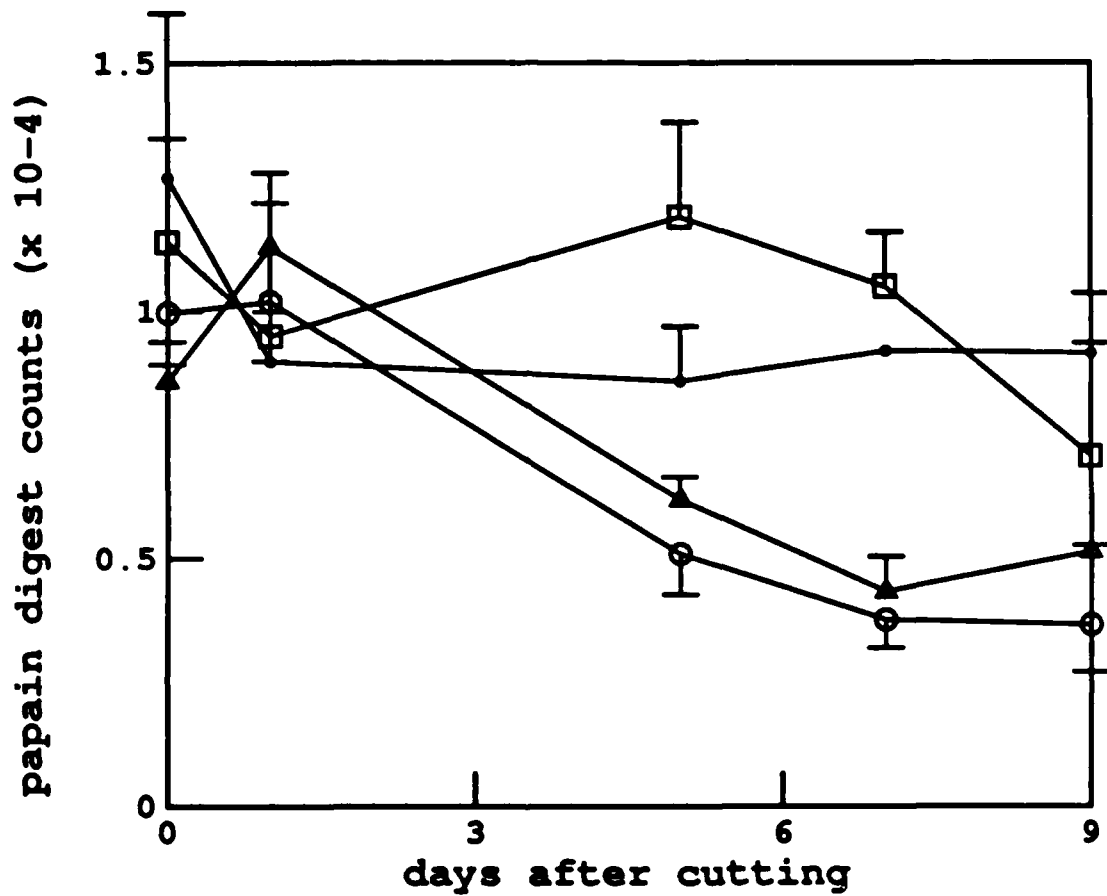


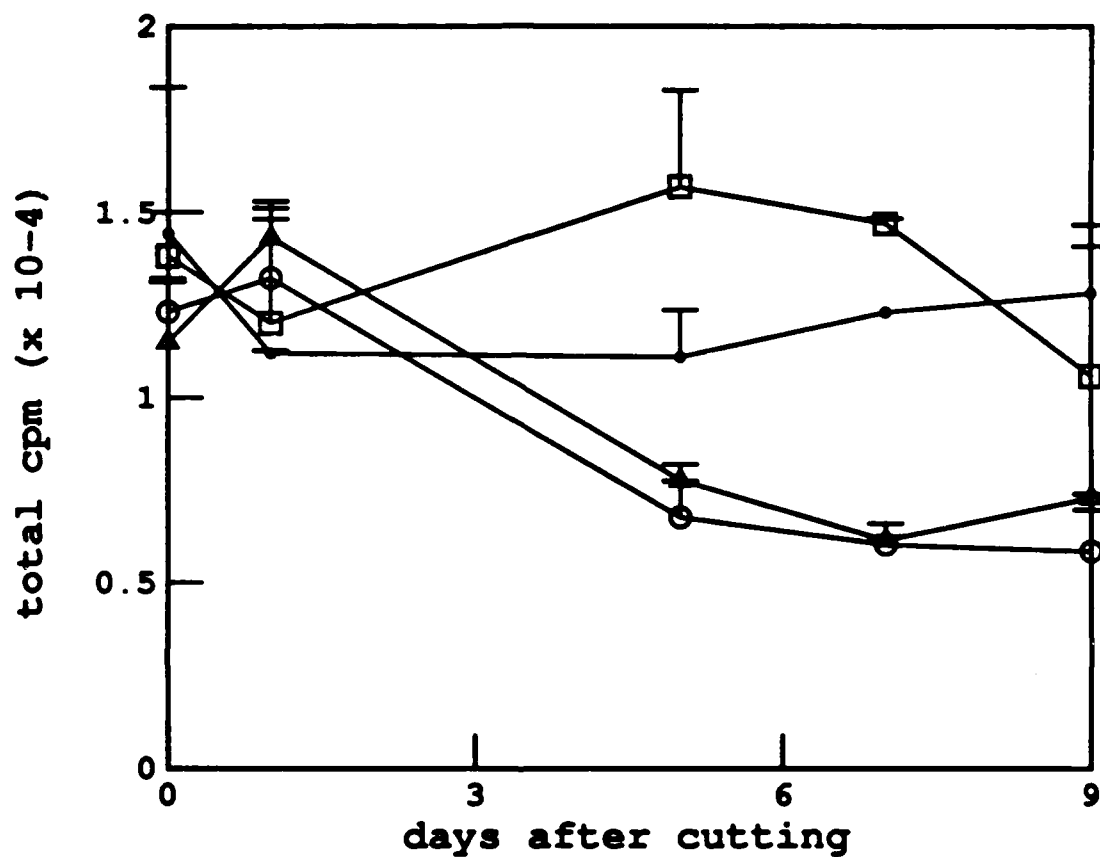
Figure 2.15: Total counts in the SDS extract, normalized to wet weight, are plotted as a function of days after tissue excision.



circles = 0.1%NS at 43 degrees  
squares = 10%FBS at 43 degrees  
triangles = 0.1%NS at 37 degrees  
dots = 10%FBS at 37 degrees

Figure 2.16: Total counts in the papain digest, normalized to wet weight, are plotted as a function of days after tissue excision.





circles = 0.1%NS at 43 degrees  
squares = 10%FBS at 43 degrees  
triangles = 0.1%NS at 37 degrees  
dots = 10%FBS at 37 degrees

Figure 2.17: Total counts in the SDS-soluble and papain digest extracts, normalized to wet weight, are plotted as a function of days after tissue excision.

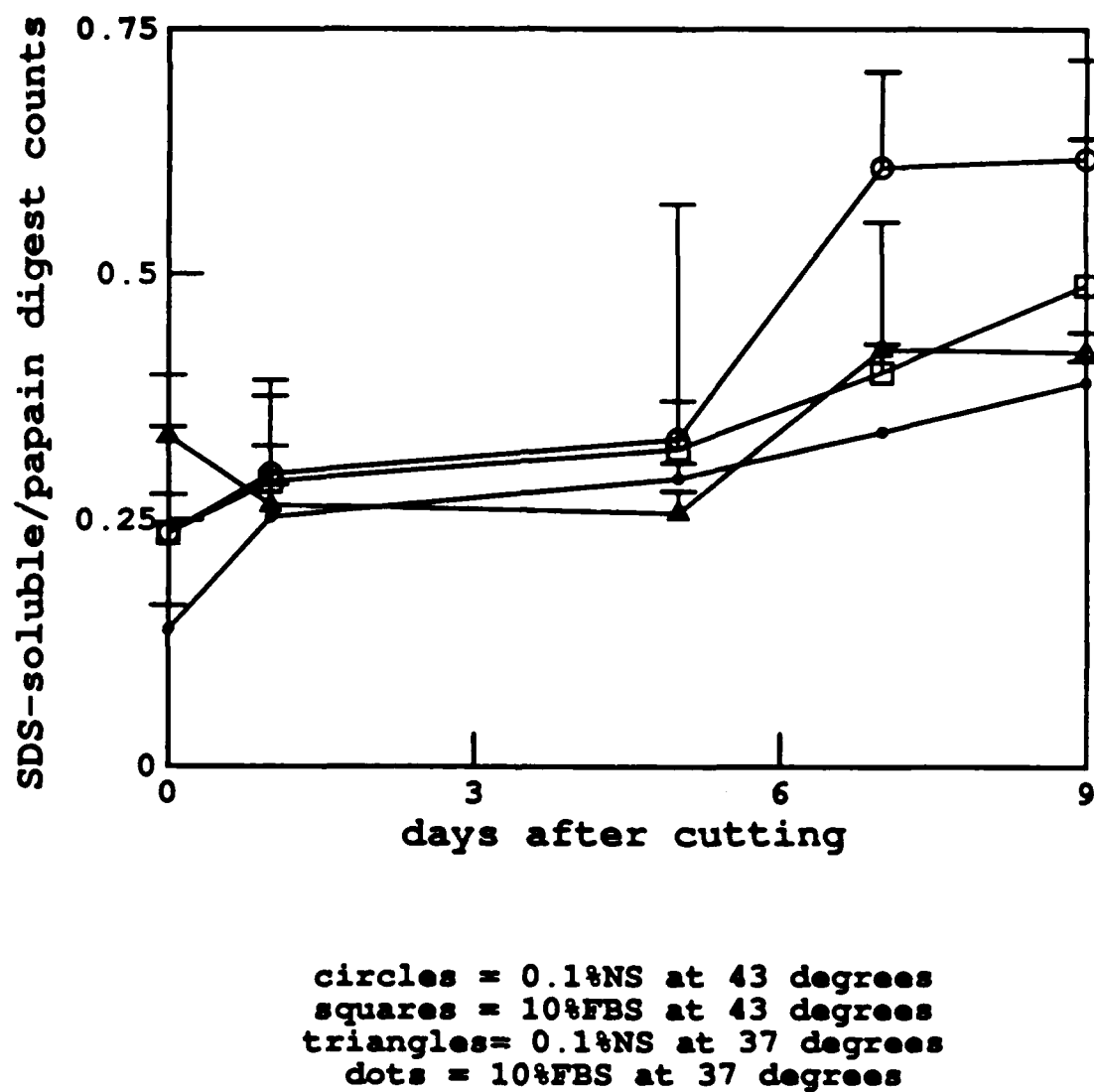


Figure 2.18: The ratio of counts in the SDS-soluble fraction to the counts in the papain digest are plotted as a function of days after tissue excision.

both fractions and the ratio of incorporation all increased.

At 45°C for 1/2 hour (days 0-5), SDS-soluble counts normalized to cell count were approximately equal in heat shocked and control plugs for days 0 and 1, were greater in controls on day 2 ( $p < 0.05$ ), and then were greater in heat shocked samples on days 3-5 (N.S,  $p < 0.005$ ,  $p < 0.002$ ), Fig. 2.19. In the 8 day experiment, in which two different plug sizes were used, incorporation into SDS-soluble proteins was greater per unit dry or wet weight for 3 mm x 2 mm plugs, than for 1/4 inch plugs. Incorporation into SDS-soluble proteins, therefore, was assessed separately for the two sizes of plugs. Consistent with the first experiment, SDS-soluble counts (Fig. 2.11) were approximately the same in heat shocked and control specimens on day 0, for both plug sizes. SDS-soluble counts were greater in heat shocked relative to control samples on all days (no significance), for 1/4 inch diameter plugs, and were greater after day 4 for 3 mm diameter plugs (no significance). (It should be noted, that the 3 mm plugs used for controls originated from positions on the joint surface that typically had higher synthetic rates than the positions from which the heat shocked plugs were excised). The increase in SDS-soluble radioactivity in heat shocked relative to control samples, in this second experiment, is consistent with the increase in the first experiment after day 2. SDS-soluble incorporation in the experiment between days 0-7d hours showed similar trends with day of excision for 0.1%NS (Fig. 2.15). Incorporation in heat shocked plugs was slightly greater than in controls for days 5-9, but the difference was not significant.

Radiolabel incorporation in the papain digest, also was different for the two different plug sizes (Fig. 2.12) in the experiment between days 0-7. Incorporation was lower in heat shocked relative to control specimens on days 2,3,5,6,7 for 1/4 inch plugs, and was lower throughout day 1-7 for 3 mm plugs (this was statistically significant at  $p < 0.05$  on day 2,6 and 7). Total incorporation (SDS and papain) similarly was lower for heat shocked 3 mm plugs relative to controls, but was nearly equal for the 1/4 inch plugs. (Fig. 2.13). In the experiment using 41°C for 3 hours,

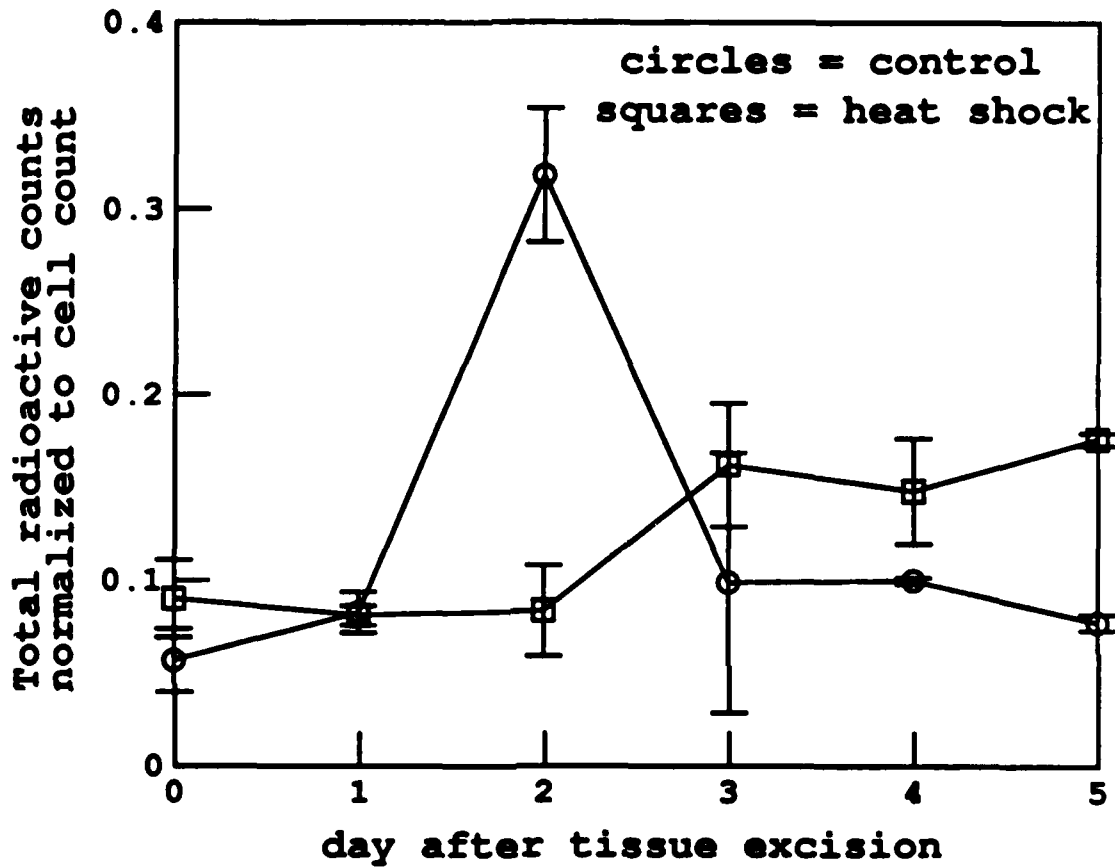


Figure 2.19: SDS-soluble counts, normalized to cell count are plotted as a function of day after tissue excision. Each point corresponds to two digested plugs. One of the plugs at each point corresponds to one lane of the gel shown in Fig. 2.9

incorporation into the papain digest and total incorporation also was lower in heat shocked relative to control plugs (no significance) between day 1 - 9.

The ratio of radiolabel in SDS-soluble to papain digest fractions was greater in plugs incubated at 43°C for 3 hours than in plugs incubated at 37°C after day 3 in culture. The increase was significant for days 5-7 ( $p < 0.005$  on day 5,  $p < 0.001$  on day 6 and 7). Unlike either the SDS-soluble counts or the papain counts alone, the ratio was independent of the plug geometry. The ratio of radiolabel was also greater in plugs incubated at 41°C for days 1 - 9 relative to plugs incubated at 37°C. The magnitude of the difference increased after day 5, and was significant on days 7 and 9 ( $p < 0.05$ ).

In both the SDS extract and papain digest, the difference between incorporation in heat shocked plugs relative to controls was greater for plugs incubated in 10%FBS compared to plugs incubated in 0.1%NS. The sign of the heat-induced change in incorporation was the same for incorporation in the SDS extract, but was reversed in the papain digest in the two serum concentrations. The difference in the ratio of SDS/papain incorporation between heated and control plugs, on the other hand, was greater in 0.1%NS than in 10%FBS.

#### **Synthesis of Procollagen and 35, 78, and 200 kD Proteins**

Although the effect of tissue excision and culture on SP70 expression and induction was not clear from visual examination of gel fluorographs from these experiments, there was a distinct qualitative change in the intensity of four other protein bands on the fluorographs for plugs incubated in 0.1%NS. The intensity of the band at 140 kD (procollagen), and 35 kD decreased on days 4 and 5 in one experiment (not discussed here) and on days 5-7 in the seven day experiment (Fig. 2.10). The intensity of the 200 and 78 kD bands increased on the same days (Fig. 2.10). The decrease in procollagen and the 35 kD protein and the increase in the intensity of the 200 and 78 kD protein were similarly seen in both heated and control plugs between day 1 and day 5 in the experiment labelling plugs on days

0,1,5,7 and 9 (Fig. 2.20 and 2.21). In this experiment, furthermore, juxtaposition of day 1 with day 5 on the gel lanes made it clear that during culture in 0.1%NS there was also a decrease in proteins of approximate apparent molecular weight 33 and 85 kD and an increase in a protein which might be the 62 kD stress protein. In 10%FBS, in contrast, no change in specific proteins with day of culture was observed. The difference in synthesis of specific proteins between the two serum concentrations after day 5 is most clearly seen in Fig. 3.20. In both 0.1%NS and 10%NS, the intensity of the band at the top of the gel, corresponding to very high molecular weight proteins (greater than 500 kD) increased with day after cutting.

## 2.4 Discussion

### 2.4.1 Protein Fractions

The SDS extract consists of intracellular proteins and proteins in the extracellular matrix that are not covalently bound and are able to diffuse out. The papain digest includes proteins that have been incorporated into the matrix or are not able to diffuse out.

The protein bands obtained on the gels using the collagenase digestion protocol were the same as the bands obtained using SDS extraction, with one exception. Since the proteins obtained from the isolated chondrocytes are primarily intracellular, this indicates that the proteins obtained in the SDS-soluble fraction that were visualized on the gels are primarily intracellular proteins. Some PGs are also solubilized in the SDS extraction. Some very high molecular weight proteins which are unable to enter the polyacrylamide separating gel are seen in fluorographs of the stacking gel (data not shown). These must be greater than 500 kD, an estimate of the maximum molecular weight protein that can enter the gel. These high molecular weight proteins may include PGs. Treadwell (personal communication) has estimated that as much as 80-85% of the PGs may be extracted by boiling in SDS. Thus the SDS-soluble fraction includes both intracellular proteins, and some

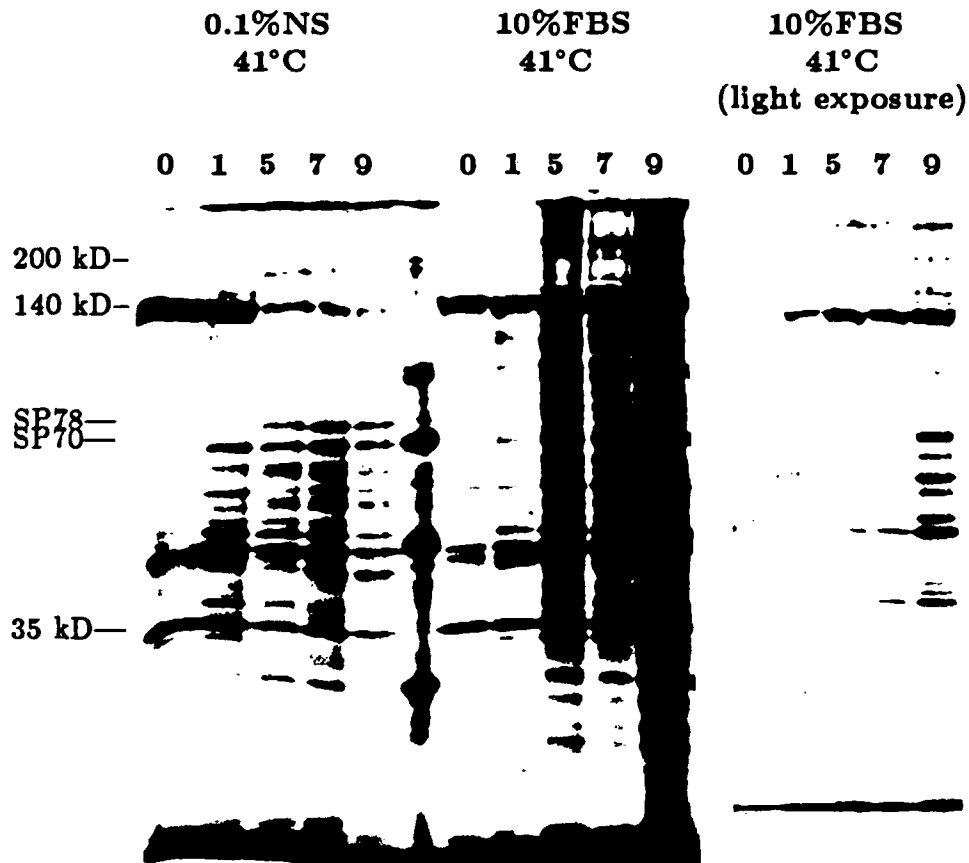


Figure 2.20: Fluorograph of cartilage plugs labelled on days 0,1,5,7 and 9 after tissue excision. Lane numbers correspond to a single plug labelled on that number day. Plugs were prepared using the SDS extraction protocol.

- (A) Plugs incubated and labelled at 43°C in 0.1%NS
- (B) Plugs incubated and labelled at 43°C in 10%FBS
- (C) Plugs incubated and labelled at 37°C in 0.1%NS

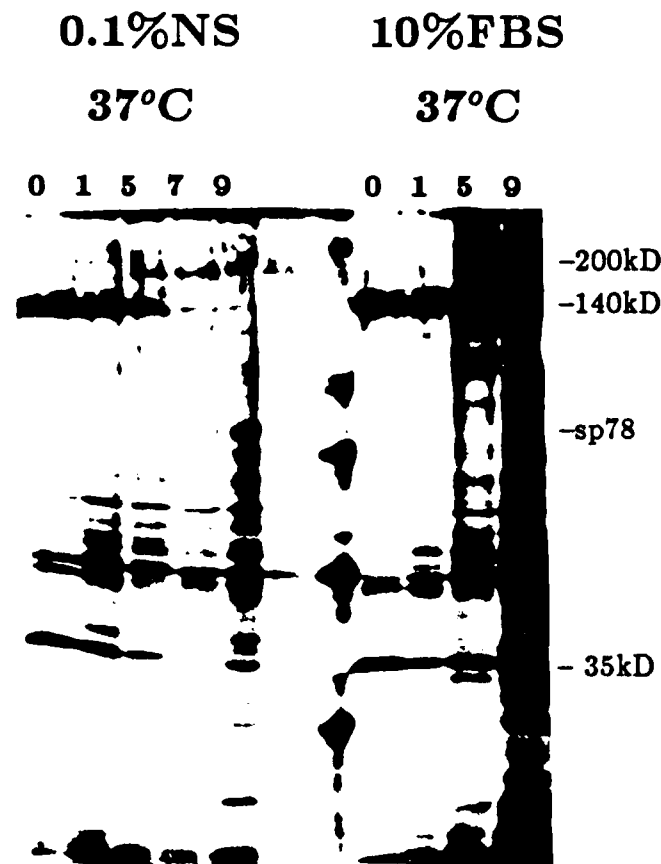


Figure 2.21: Fluorograph of cartilage plugs labelled on days 0,1,5,7 and 9 after tissue excision. Lane numbers correspond to a single plug labelled on that number day. Plugs were prepared using the SDS extraction protocol.  
(A) Plugs incubated and labelled at 37°C in 0.1%NS  
(B) Plugs incubated and labelled at 37°C in 10%FBS



extracellular proteins which are not covalently bound in the matrix.

The papain digest includes proteins which are covalently bound in the matrix, or do not diffuse out of the matrix. Collagen is one major extracellular protein that is covalently bound in the matrix. Fibronectin can also bind covalently to collagen [79]. Approximately half of the fibronectin in confluent cell cultures of human or hamster fibroblasts is insoluble in SDS under reducing conditions. Those PGs not extracted by SDS will also be included in the papain digest.

#### **2.4.2 Stress Response**

A strong induction by incubation at temperatures equal to or greater than 41°C and the lack of stress protein induction at 37.5°C and 38.5°C, is consistent with the findings of previous investigators [132,131,111]. Madreperla et al. [131] found that SP70 was not induced in chondrocytes by incubation at 38°C but was induced by incubation at 40°C (for 45 min). Kubo et al. [111] found that SP70 was induced by incubation at temperatures greater than 39°C (for 90 minutes). The less intense stress response to incubation at 43°C by plugs pre-incubated at 38.5-39°C relative to controls, however, might suggest that chondrocytes do synthesize low levels of SP70 (not observable on the gels), thus reducing the amount of newly synthesized SP70 required to respond to the more severe stress [6,5,125].

The lack of SP110 induction at temperatures less than 45°C is not inconsistent with the findings of previous investigators. Madreperla et al. [131] observed no SP110 synthesis for temperatures below 45°C. Kubo et al. [111] observed inconsistent SP110 synthesis for temperatures greater than 40°C.

Stress proteins other than SP70 and SP110 had been observed in chondrocyte cell culture [200], but not reported in the literature. However, Madreperla had noted the position of a 77 and 92 kD protein on some gels. Although he did not designate them as stress proteins, examination of the published gels suggests that they were indeed enhanced by heat shock. Thus the 77 kD and 92 kD protein bands noted

by *Madreperla* may correspond to the 78 kD and 90 kD protein bands enhanced by heat in this tissue culture system. The molecular weights of many of the proteins enhanced by heat shock in this tissue culture system are similar to molecular weights reported in the literature for heat induced proteins in other mammalian cell and tissue types (see Table 2.3).

In terms of synthesis of other proteins, the response to heat stress in cartilage organ culture presents some unique aspects when compared to stress response in other tissue types. These include: a specific decrease in the intensity of the procollagen band in cartilage samples incubated at elevated temperatures before day 3, relative to controls; a small increase in total SDS-soluble protein synthesis in heat shocked relative to control specimens and an increase in incorporation in the papain digest over short times; and an increase in the ratio of SDS-soluble to papain digest fractions (especially after day 4) over long times. Synthesis of stress proteins in other tissue and cell types has been accompanied by either little change or by a decrease in total protein synthesis [5,107,42,91,208,125,122].

The decrease in the intensity of the procollagen band and in radiolabel incorporation in the papain digest for long incubation times at elevated temperature may be related. Since one of the major proteins which is covalently bound in the matrix is collagen, the decrease in the amount of protein bound in the matrix (the papain digest), during the labelling period may be at least partially due to a decline in procollagen synthesis, as represented by the lower intensity of the procollagen band corresponding to heat shocked plugs. Thus, the decrease in the intensity of the procollagen band and the decrease in  $^{35}\text{S}$ -methionine incorporation in the papain digest is consistent with a decline in protein synthesis, in agreement with the results of previous work showing a decline in synthesis with heat shock. However, it is a more specific response, in that only incorporation in extracellular matrix proteins which are covalently bound to the matrix appears to decrease (while incorporation into SDS-soluble proteins which are not bound to the matrix increases).

Cartilage organ culture (this thesis)	Cartilage cell culture [132,131]	Hamster fibroblasts [124]		Chinese Hamster ovary cells [193]		Rat tissue culture	HeLa cells
		major	minor	major	minor		
97	110	97		110			110 [196,208]
90		87		89			100 [196,208,28], 95 [48]
78					76		90 [196,208], 85-90 [185]
70	70	70		68		74 [25]	80 [196,208,91]
62					66	71 [92,209]	72-74 [28], 72/73 [196,208]
57			59				70 [185,91], 68 [48]
48							
41			31				37 [28]
			26				27 [91]

Table 2.3: The molecular weight of stress proteins found in the cartilage organ culture used for this thesis compared to molecular weights reported in the literature for mammalian stress proteins. The references are listed in the Bibliography.

An alternative explanation for the decrease in the intensity of the procollagen band in heat shocked samples might be an increase in degradation rather than a decrease in synthesis. Wooley [214] showed that an increase in temperature of 6°C (from 30 to 36°C) resulted in a 10 fold increase in degradation of collagen fibrils with synovial collagenase. He attributes the increase in degradation to reduction of fibril stability.

In isolated chondrocytes, Madreperla et al. reported that incubation at elevated temperatures was accompanied by an increase in protein synthesis for temperatures up to 40°C. The increase in SDS-soluble synthesis observed in this tissue culture system is consistent with the response observed in isolated chondrocytes for small increases in temperature. For isolated chondrocytes, the total protein synthesis measured is predominantly intracellular, while the SDS extract obtained here includes intracellular proteins with some fraction of the proteins secreted into the extracellular space. Since the intracellular proteins include the stress proteins, the increase in incorporation in the SDS-soluble fraction might be attributed to a large increase in stress protein synthesis superimposed on a small decrease in overall normal protein synthesis. In this case, the decrease in normal protein synthesis and associated decrease in secretion of extracellular proteins into the matrix would result in a decrease in the amount available to be bound to the matrix. Thus, over a long enough time, the decrease in secreted protein would in turn result in less incorporation in the bound extracellular fraction which is included in the papain digest. Taken together, the small increase in the fraction including intracellular proteins and the decrease in incorporation in bound extracellular proteins over long times (greater than 2 hours) may represent an overall decrease in normal protein synthesis accompanied by an increase in stress protein synthesis.

For short times, (less than 2 hours), the increase in the papain digest might not be consistent with this interpretation. For short times, the data is consistent with either an increase in total synthesis, a decrease in degradation in the matrix, or

accelerated binding of proteins within the matrix. A change in temperature might increase the activity of the enzymes involved in binding proteins in the matrix. Then for short exposures to heat, normal protein synthesis may not decline sufficiently to reduce the pool of extracellular proteins available for binding.

### **2.4.3 Alterations in Protein Synthesis With Time in Culture**

#### **Total Protein Synthesis**

The choice of concentration of NS in the medium (0.1%NS) was motivated by control studies [72,177,178] showing that cartilage samples incubated in 0.1% NS achieved a steady rate of protein synthesis after several days in culture, and that the steady state rate was lower at 0.1% NS than at higher concentrations (1, 5 or 10%). This is also consistent with the work of Handley et al. [81] and Hascall et al. [82] which showed that the level of steady state proteoglycan synthesis after several days in culture is governed in part by serum concentration in the medium. Thus, since the rate of synthesis was neither at a minimum or maximum rate, either increases or decreases in total synthesis due to incubation at elevated temperatures or to an electric field might be observed.

Changes in total protein synthesis with time in tissue culture are similar to the results of studies by other investigators in low serum concentrations [177,178, 72], in that after day 1 total protein synthesis decreased to a steady state level of synthesis below the day 0 level by about 4 days. The level of proteoglycan synthesis in culture in studies by other investigators also has a similar pattern with time in culture [81,82,177,178,72] at low serum concentrations.

In 10%FBS, little change in total protein synthesis was observed throughout the culture period. Handley et al. [81] also found little change in sulfate incorporation in cartilage explants cultured using this serum concentration. They reported that sulfate incorporation in cartilage explants maintained in 10%FCS declined very slowly (15% over 9 days) to day 9. However, other investigators found that PG syn-

thesis varied with day of culture in 10%FCS. Lane and Brighton [115] found that sulfate incorporation increased in 10%FCS, and McKenzie et al. [137] had reported an initial increase (on day 1), followed by a decline to day 3. In the same experiment, McKenzie et al. reported that protein synthesis ( $^3\text{H}$ -leucine incorporation) also first increased, and then decreased over 3 days. However, McKenzie et al. used stainless steel grids to support their samples, and these stainless steel grids may have produced reaction products in the media which might have altered PG and protein synthesis.

The difference between the way in which the two protein fractions (SDS-soluble and papain digest) vary with day after excision, however, had not previously been investigated. The difference in synthetic rate with day of culture between the two fractions may be associated with chondrocyte adjustment to the change in the external environment from *in vivo* to the specific *in vitro* conditions. In 0.1% NS the upturn in the ratio of SDS/papain incorporation occurred on approximately the same days as changes in specific proteins (procollagen, and the 200 kD, 78 kD and 35 kD proteins). The increase in the ratio, therefore, may be associated with a specific decrease in the synthesis of proteins which are transported to and then bound in the extracellular matrix. In 10%FBS the upturn was not accompanied by any apparent changes in specific proteins. The results in 10%FBS compared to 0.1%NS suggest that either cartilage maintained in 10%FBS remains more metabolically active in the richer environment, or that the 10%FBS creates an *in vitro* environment which indicates greater accumulation of extracellular matrix. In the latter case, culture in 10% FBS might delay, rather than eliminate a change in the proportion of intracellular proteins synthesized relative to the proteins synthesized, transported and then bound to the matrix. The change in SDS/papain incorporation did not reach a steady state by day 9 in 10%FBS, as it did in 0.1%NS. It is possible that over longer culture periods, both the change in ratio would reach a steady state, and the specific changes in proteins would occur. The work of Handley et al. [81],

however, suggests, that the concentration of serum sets the level of metabolic activity. They showed that cartilage explants maintained in culture adjusted to increases or decreases in serum concentration by increases or decreases respectively in sulfate incorporation for up to 21 days in culture.

The difference between the two plug sizes in radioactivity in the SDS-soluble and papain digest fractions is most likely associated with differences in diffusion times associated with the plug geometries. Differences in radiolabel incorporation over a cartilage plug thickness have been observed previously [178]. Maroudas [135] calculated that the time to reach 90% of the final equilibrium concentration of radiolabel ( $^{35}\text{S}$ -sulfate) within a 2 mm thick cartilage plug exposed to label on both facets is on the order of 0.5 hours. This is a significant fraction of the total labelling time (3 hours) used in this experiment. The ratio between SDS-soluble and papain digest fractions is not expected to depend on diffusion rates within each plug, and therefore, is independent of plug geometry.

#### **Synthesis of Collagen and 35,78 and 200 kD Proteins**

Changes in the synthesis of some specific proteins (200,140,78, and 35 kD) during culture in 0.1%NS might be attributed to the chondrocytes modifying the extracellular matrix in response to the changed environment of specific *in vitro* conditions vs. *in vivo* conditions, especially since the changes occur in the synthesis of proteins which may be associated with the extracellular matrix. The 140 kD protein was identified as procollagen. The 200 and 35 kD proteins may also be extracellular proteins that have not been incorporated into the matrix. The molecular weight of the 200 kD protein is within calibration error of the the apparent molecular weights of the fibronectin subunits (210 and 230 kD) for high molecular weight proteins on these 10% gels. Furthermore, the fibronectin subunits should appear as a doublet and the 200 kD protein does appear as a doublet with sufficiently light autoradiographic exposures. 35 kD is the molecular weight of the collagen C-propeptide end.

Wooley [214] has shown that collagenase increases in serum-free culture of skin tissue explants. Thus, the decrease in the intensity of the procollagen band with day after excision might be associated either with a decrease in procollagen synthesis, or with an increase in procollagen degradation by native collagenase.

Miller et al. [147] suggested that chondrocytes synthesize fibronectin in response to loss of extracellular matrix. Transformed chondrocytes also synthesize a fibronectin-like molecule and decreased amounts of Type II collagen [201].

The total content of collagen C-propeptide (as well as helical collagen content) has previously been shown to vary in culture of chondrocytes isolated from epiphyseal, growth plate, and nasal cartilage [95]. In epiphyseal cartilage cell culture, for example, C-propeptide content and helical collagen content, normalized to DNA content, decreased slightly over 5 days in culture, or first increased and then decreased over days 1-5, respectively. However, since these measurements were made in cell, rather than organ culture, and the culture medium was supplemented with a higher concentration of serum (10%) with less frequent media changes (3 day), it is difficult to compare these results with the changes observed in this organ culture system using 0.1%NS.

The apparent increase in the 78 kD protein is intriguing, since this protein is also induced in response to heat. The protein at approximately 62 kD which increases between day 1 and day 5 may also be a stress protein. In mammalian cells the stress protein of apparent molecular weight near 80 kD has been identified as a glucose-regulated protein [40,92]. That is, synthesis increases in glucose deprived media, or in the presence of inhibitors of protein glycosylation. DMEM is high in glucose, so the cartilage plugs are not glucose deprived. However, the increase of the 78 kD protein with time in culture raises the issue of possible limits on other metabolic factors in glucose utilization.

In fact, in several ways the response of cartilage plugs incubated in 0.1%NS for greater than 5 days, resembles the stress response, but with two important dif-



ferences. As in the stress response, SP78 increases (and possibly the 62 kD protein), procollagen synthesis decreases, and total protein synthesis decreases. The differences are that incorporation in the SDS extract also decreases, and that synthesis of SP70 in response to heat is enhanced. Synthesis of SP70 in heated plugs is greater on day 5 than on day 0 in culture using 0.1%NS and qualitatively is greater on day 5 in culture using 0.1%NS than in culture using 10%FBS.

In 10%FBS the specific changes in protein synthesis were not observed. The lack of change may be reflected in the ratio of SDS/papain incorporation, which is lower in 10%FBS than in 0.1%NS after day 5, as discussed previously. The lack of change may indicate that the in vitro conditions are more favorable to the chondrocytes, or more similar to in vivo conditions, or that the conditions delay adaptation to the new environment.

### **Stress Response**

Hightower and White [14,15] suggested that stress protein synthesis may be a response to tissue wounding and trauma. The induction of SP70 following tissue excision in the first experiment suggests that cartilage does have a stress response to tissue excision. The apparent increase in the intensity of the heat induced stress response (45°C for 1/2 hour) over the first few days might similarly reflect a low level stress response to excision, which reduces the amount of SP70 synthesis required to respond to the heat induced stress. Similarly, in the experiment at 41°C, the increase in intensity of stress protein synthesis from day 0 - day 1 may indicate a stress response to excision on day 0. The constant intensity of the SP70 band with day after excision in the experiment using 43°C for 3 hours, may not reflect a lack of excision induced stress response, but rather that the response to the more severe stress (a higher temperature relative to the 41°C experiment, and a longer incubation time relative to the 45°C experiment) requires a higher intracellular concentration of SP70.

The difference in total radioactivity in SDS-soluble and papain digest between heat shocked and control samples became more distinct following 4 days in culture in both 0.1%NS and in 10%FBS. This increase in the magnitude of the heat-induced change in synthesis with time in culture may have to do both with an excision induced stress response in the first few days, and with the tissue adaptation to culture conditions. An excision induced stress response would make the control plugs (which are stressed by excision) more similar to the experimental plugs (stressed by excision and heat). Tissue adaptation to culture conditions might lead to smaller differences in the biosynthetic behavior of plugs from different positions of origin on the joint surface, so that the signal to noise ratio for observing changes in biosynthetic behavior is increased.

## 2.5 Stress Response and Total Protein Synthesis Assay for Electric Field Effects

The lack of apparent stress response to temperatures of less than 38.5°C is important, since it implies that *significant* ohmic heating would have to occur during electrical stimulation, before SP70 synthesis is induced by heating rather than a direct field effect. Maximum temperatures of approximately 38°C were calculated for current densities of 50 mA/cm<sup>2</sup> in the electric exposure system when the temperature control system is not used. They were measured to be less than 0.2°C at 30 mA/cm<sup>2</sup> (see Chapter III). The less intense stress response to incubation at 43°C by plugs pre-incubated at 38.5-39°C relative to controls, however, does suggest that at 38.5-39°C chondrocytes do synthesize low levels of SP70 (not observable on the gels), thus reducing the amount of newly synthesized SP70 required to respond to the more severe stress. [6,5,125].

A serum concentration of 0.1%NS was chosen, since synthesis at this concentration was at an intermediate level, and therefore is subject to field induced increases or decreases in synthesis. Comparison to 10%FBS showed that synthesis was less than maximal. Exposure to heat showed that synthesis could be stimulated or depressed in 0.1%NS, for short or long exposure times, respectively. In addition, it was observed that the SP70 band relative to normal protein bands was qualitatively more intense for plugs incubated in 0.1%NS relative to plugs incubated in 10%FBS.

On the basis of the observed dependence of the stress response and total protein synthesis with day after cutting in 0.1%NS, electrical stimulation experiments should be done on day 5 or later after cutting.

Sensitivity to heating was greatest after day 4. The stress response induced by cutting in one control experiment had apparently been eliminated after day 4. The intensity of the stress response induced by heating at 45°C for 1/2 hour or by heating at 41°C for 3 hours had increased after the first day. The change in

the ratio of SDS-soluble/papain digest incorporation that was induced by heat also increased after day 4.

The changes in incorporation in the SDS extract and papain digest with day after excision suggested that experiments be done after day 5 so that protein synthesis is in steady state. SDS-soluble protein synthesis reached a steady rate 2-3 days after cutting. The radioactivity in the papain digest similarly supports this conclusion since this also reaches a nearly steady state by approximately day 4 for plugs incubated at 37°C. The ratio of incorporation in the SDS-soluble extract relative to the papain digest reached a nearly steady state after day 5.

Changes in the synthesis of specific proteins (200, 140, 78, and 35 kD), assessed qualitatively from the intensity of the protein bands on the gel, suggest that experiments should be done after day 4. The changes may be due to the chondrocytes adapting to the changed environment of *in vitro* versus *in vivo*. Loss of cell viability seems unlikely since total SDS-soluble protein synthesis does not decrease further after day 2, and only certain proteins show the change with time after cutting.

In experiments lasting over 1 week, <sup>35</sup>S-methionine incorporation in the papain digest appeared to decline somewhat over 1 week, especially in heat shocked plugs. If the decline in ratio is associated with completion of adaptation to the new conditions (since three of the proteins may be matrix proteins), this decline in protein synthesis may not be important.

In summary, control experiments have shown that

- (1) During electrical stimulation, ohmic heating is not expected to give rise to stress response for less than a 2°C increase in temperature in the stimulated chamber relative to the control (a temperature increase neither calculated, nor measured).

- (2) Cartilage plugs cultured in 0.1%NS had rates of total protein synthesis that were less than rates of plugs incubated in 10%FBS and less than maximal. Cartilage specimens incubated in 0.1%NS could therefore exhibit enhanced synthesis

to a possible stimulatory effect by electric fields on protein synthesis. It was shown that both stimulation or reduction in synthetic rates was measurable, using heat as the stimulus for short and long times, respectively. Further, culture in 0.1%NS appeared to increase the intensity of stress protein induction in plugs relative to culture in 10%FBS.

(3) To obtain a consistent, steady state, control level of protein synthesis, and to maximize the sensitivity of the stress response to a stimulus such as exposure to electric fields, experiments are best done after day 5. Experiments were done before day 9, in order to remain within the period investigated in the control studies.

## Chapter III

### Experimental Model: Electrical Stimulation Experiments

In chapter I, stress protein synthesis and total protein synthesis in cartilage tissue was proposed as an experimental model for electric field interaction with biological tissue. Induction of stress proteins was shown not to be induced in response by a variety of agents which act on cellular processes to produce a toxic accumulation of abnormal proteins. While induction of stress protein synthesis is not mechanism specific, the particular kinetics and the particular stress proteins induced are specific to the type of inducing agent. Therefore, in the event that fields induce stress protein synthesis, the field induced pattern of synthesis might be compared to the pattern of synthesis produced by other agents. Changes in the synthesis of normal proteins are a part of the stress response. In cartilage, changes in synthesis of normal proteins also occur in response to other changes in state (e.g. in the response to organ culture, or to mechanical stimulation [71,179]). Total protein synthesis, therefore, is another means of investigating field induced changes in metabolic state.

Cartilage specimens were exposed to current densities between a few mA/cm<sup>2</sup> and 30 mA/cm<sup>2</sup>, at frequencies of 1, 10, and 100 Hz and 1 and 10 kHz. This range of field magnitudes and frequencies was chosen based on (1) the fields induced in cartilage by physiological mechanical loading (frequencies between dc and a few kHz and current densities as large as a few mA/cm<sup>2</sup>) and (2) the field magnitudes and frequencies for which the model mechanisms described in Chapter I predict a change in physiological parameters on the order of 1-10% (frequencies less than 100 kHz and current densities on the order of mA/cm<sup>2</sup>). The physical interaction mechanisms were based on linear models, and assumed no amplification due to non-linear biological processes. Therefore, these models may give conservative (high) estimates for the current densities that might be important. Thus, although current densities of 1-10 mA/cm<sup>2</sup> were used as the initial starting point, the experimental

model does not exclude the possibility of observing field effects on synthesis which might be induced by lower current densities.

Culture conditions for the cartilage tissue were chosen, based on the control studies described in chapter II, with a view to optimizing the sensitivity of stress and total protein synthesis to a stimulus such as the electric field, and to produce steady state protein synthesis prior to electrical stimulation. Steady state protein synthesis was characterized by the absence of stress protein synthesis and of changes in other specific proteins over several days, and by rates of total protein synthesis that were approximately constant over several days. Additional control studies were done to develop protocols for labelling cartilage plugs and subsequent preparation of the cartilage samples for biochemical analysis (extraction of SDS-soluble molecules, followed by gel electrophoresis and papain digestion of the residual plug).

The response to heat, a stimulus known to produce a stress response was used to examine the sensitivity of the cartilage plugs to a stimulus under different culture conditions—different serum concentrations and days of culture. Stress proteins were shown to be induced by heat and were visualized on gel fluorographs. The intensity of the stress protein bands on the fluorographs depended on the temperature and length of incubation. Both stress response and total protein synthesis were assessed as a function of day after tissue excision in culture using serum concentrations of 0.1%NS or 10%FBS. A serum concentration of 0.1% NS was chosen since at this concentration, a steady rate of total protein synthesis was achieved after several days that was neither a maximum or minimum rate. Thus, under these conditions, it should be possible to measure if exposure to electric fields could either increase or decrease total protein synthesis. In 0.1%NS, total protein synthesis, synthesis of some specific proteins, including procollagen, and synthesis of stress proteins were found to reach a nearly steady state by day 5 after cutting. Consequently, all electrical experiments were done on or after day 5, when synthesis by plugs not stimulated by electric fields had reached this steady state.

In this chapter electrical stimulation experiments and their results are described. Discussion of these results is reserved for the following chapter. Cylindrical cartilage disks were stimulated by electric fields for 12 hours in a chamber filled with media containing  $^{35}\text{S}$ -methionine. Unstimulated controls were incubated and labelled in an identical specimen chamber in the same incubator. Protein synthesis in electrically stimulated specimens relative to controls was assessed. Stress protein (specifically SP70) synthesis was assessed by examination of gel fluorographs. Total protein synthesis was assessed by counting the total radioactive label incorporated into proteins extracted by SDS (which included the gel sample buffer solution and SDS washes) and label incorporated into proteins in the papain digest of the residual plug.

### **3.1 Experimental Protocol: Materials and Methods**

#### **3.1.1 Specimen preparation**

Cartilage specimens were prepared following the protocol described previously (Chapter II). For the studies reported here, 3 mm diameter and 2 mm thick plugs were used. Specimens were maintained in DMEM supplemented with 0.1%NS (and antibiotics for the first 2 days), except where noted. Media was changed every two days.

#### **3.1.2 Stimulation chambers**

The chamber consisted of pairs of cylindrical teflon tubes filled with media (Fig. 3.1). These pairs could be assembled in two configurations. Either the platinum electrodes capped each end of each tube (for experiments using 6 control and 6 experimental plugs), or platinum electrodes capped one end of each tube and the tubes were connected at their other ends by tygon tubing (for experiments using 12 experimental and 12 control plugs). Each teflon tube was supported in a rectangular pan by six aluminum and stainless steel braces. Cartilage plugs were placed in



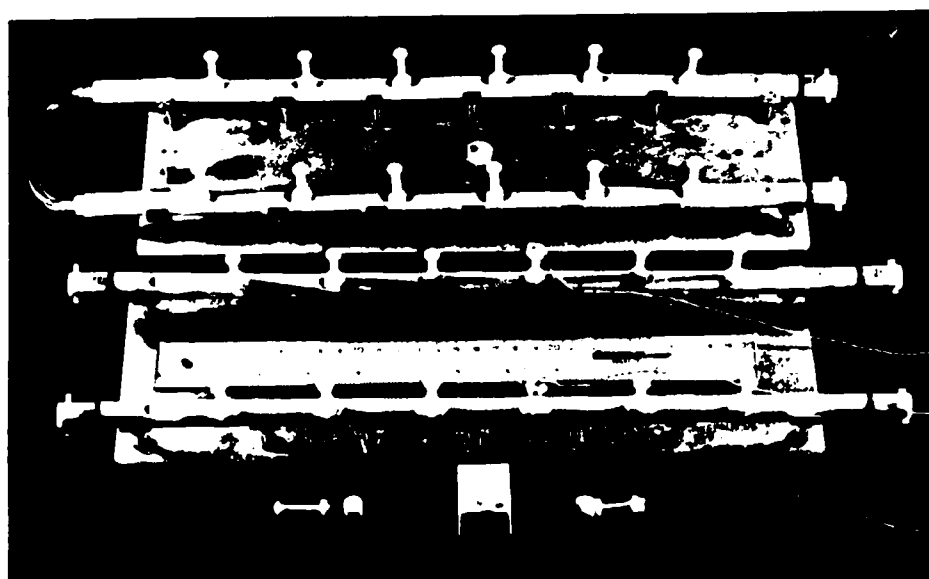
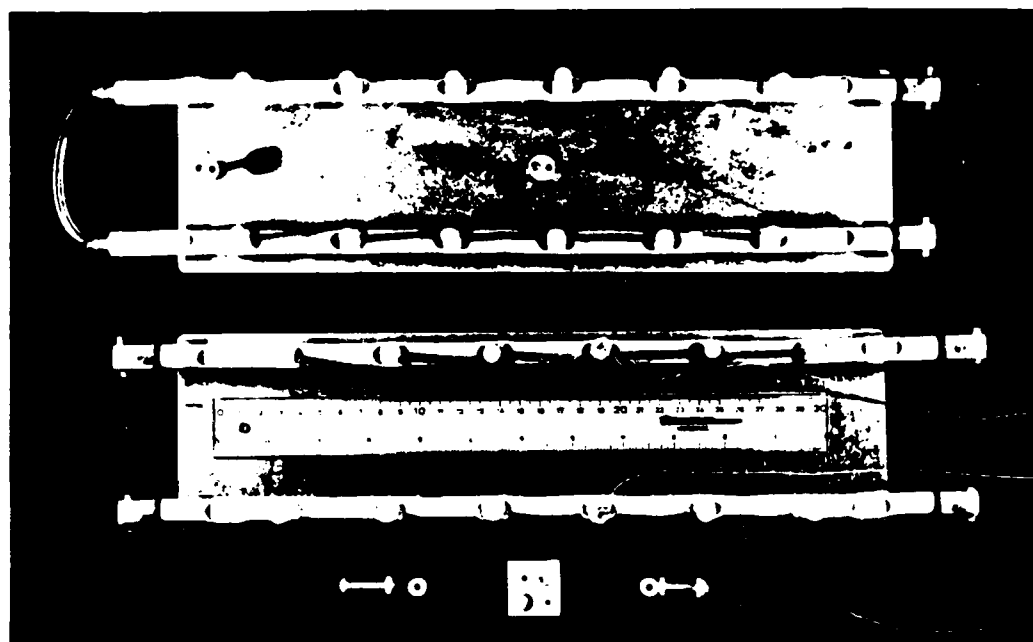


Figure 3.1: The electrical stimulation chamber, assembled with cartilage and agarose plug holders in place.

Figure 3.1: The electrical stimulation chamber, assembled with cartilage and agarose plug holders in place.

cylindrical teflon holders. The holders were then individually inserted through slots in the tubes, so that they were concentric to the long axis of the tube. Currents were applied to the six or twelve specimens in series by means of the cylindrical platinum electrodes at each end of the tube. The cartilage plugs were separated from the electrodes by a 1.5 cm length of agarose in a cylindrical teflon holder to impose a barrier to convective transport of electrode reaction products during a 12 hour electrical exposure (see Appendix C.2 for control studies of agarose toxicity). The tubes were designed to be half submerged in a waterbath in order to maintain a constant temperature at high current densities, if required.

The chambers were prepared by soaking in a detergent solution (several hours minimum), scrubbing and rinsing thoroughly with tap and then distilled water, and then soaking in distilled water for 1 hour. The chambers were then assembled and autoclaved. Agarose plugs (2 % (w/v) agarose (SEAKEM,NKT from FMC) in DMEM with no methionine) were cast under sterile conditions prior to loading the cartilage specimens into the chamber.

### **3.1.3 <sup>35</sup>S-methionine Incorporation During Electrical Stimulation**

Electrical experiments were done on or after day 5, and before day 8 post excision. Plugs were labelled in DMEM-N (Chapter II, Methods) with 0.1 mM methionine and 5 $\mu$ Ci/ml <sup>35</sup>S-methionine for 12 hours during electrical stimulation. Cartilage plugs were first inserted into the cartilage holders with forceps, and the holders were then placed in the chamber. (Control plugs were matched to experimental plugs by core position on the joint surface). Agarose holders were inserted at the ends adjacent to the electrode and labelling media was then pipetted into the chamber so that the cartilage plugs were completely covered. The resistance between the electrodes was measured for both experimental and control chambers to check for the presence of bubbles in the media, especially near the cartilage plugs in the cartilage holders. The media filled chamber was then placed in the incubator (37°C,

5% CO<sub>2</sub>) and the electrodes were connected to the power supply.

After stimulation for 12 hours at a given frequency and current density, the chamber was removed from the incubator. As a routine test for electrode reaction products, 1 ml of media was removed from the two holder positions closest to each electrode in the experimental chamber, pipetted into a 24 well culture dish and used to incubate and label fresh plugs, matched to controls incubated and labelled in previously unused media. Following a 12 hour incubation, these control plugs were processed as described below for the electrically stimulated plugs and controls. Media from all positions in the chamber was plated on blood agar plates (Gibco, Brucella agar with 5% horse blood), and the plates were placed in a bacteriological incubator and checked three days later for bacterial growth. Following plating, the media was aspirated out of the chamber. The cartilage holders were removed, and the cartilage plugs were pushed gently out of the holders into a 24 well dish. Cartilage plugs were chased (1 hour minimum incubation in culture dishes with 1 ml DMEM containing 2 mM cold methionine) and then washed in PBS, weighed and processed as described earlier. All experiments routinely included a "culture dish control" at both 37 and 43°C. The culture dish control at 37°C tested for possible synthesis of SP70 in control or electrically stimulated plugs due to naturally occurring SP70 synthesis by the chondrocytes of a particular cow joint, or due to viral infection. The culture dish control at 43°C served to confirm that the chondrocytes of a particular cow joint were able to synthesize SP70.

### **Assays for Protein Synthesis**

Samples for SDS-gel electrophoresis were obtained from each plug as described previously (Chapter II). Aliquots of each gel sample and of the SDS washes were used to assess incorporation of <sup>35</sup>S-methionine into SDS-soluble molecules. Following papain digestion of the residual plug, an aliquot of the papain digest was also counted.

## **3.2 Control Studies**

### **3.2.1 Introduction**

In order to assess the effect of the electric field on protein synthesis, it was important to eliminate other possible sources of artifactual changes in synthesis. Temperature in the chamber was first measured in order to determine if ohmic heating due to the electric field might induce changes in synthesis (section following). The pattern of protein synthesis was not altered by incubation in the chamber in the absence of applied current densities (Appendix C.1). Protein bands on gel fluorographs were the same for cartilage specimens incubated in the chamber and for cartilage specimens incubated in culture dishes. Pilot electrical stimulation studies (Appendix C.3) pointed up a need for careful preparation of the chamber to eliminate possible induction of SP70 by contamination, and for control studies of SP70 induction and changes in total protein synthesis due to electrode reaction products (described below).

### **3.2.2 Induction of SP70 by Electrode Reaction Products**

In the electrical studies reported here, holders filled with agarose were used to separate the cartilage samples from the electrodes. In some pilot experiments, in which agarose bridges were not used, stress proteins were induced in plugs located at one or both ends of the chamber. To test the hypothesis that this was due to electrode reaction products, rather than a direct field effect, control experiments were done in which media was removed from each position of the chamber following an electrical experiment, and used to culture fresh plugs. SP70 was induced in fresh plugs incubated in media from the ends of the chamber, confirming the hypothesis that electrode reaction products were responsible. In addition, total protein synthesis by plugs incubated in media obtained near the electrodes decreased relative to controls plugs.

Three control experiments were done following electrical experiments to explore the effects of electrode reaction products, and the effectiveness of the agarose plugs in eliminating these effects. One ml of media was taken from the media adjacent to each cartilage specimen and adjacent to the electrode after each electrical experiment. Protein synthesis by plugs cultured in 24 well culture dishes for 6 hours in media taken from each position in the electrical chamber (conditioned media) was compared to synthesis by matched plugs in fresh media. Conditioned media was obtained following (1) an electrical experiment in which no agarose plugs were inserted between the electrodes and the cartilage holders during stimulation by  $2 \text{ mA/cm}^2$  (1/4 inch plugs) at 10 Hz for 6 hours and (2) two electrical experiments in which agarose plugs were used during stimulation by  $6 \text{ mA/cm}^2$  (1/4 inch plugs) at 10 Hz for 12 hours, and  $30 \text{ mA/cm}^2$  (3 mm plugs) at 100 Hz for 12 hours. In the first experiment ( $2 \text{ mA/cm}^2$  at 10 Hz, with no agarose plugs), electrically stimulated cartilage samples at both ends of the experimental chamber synthesized SP70, while their matched controls did not (Fig. 3.2). Cartilage plugs incubated in the conditioned media from the end positions of the experimental chamber also exhibited SP70 synthesis relative to controls (Fig. 3.3). In the two electrical experiments in which agarose bridges were used, no SP70 synthesis was observed in electrically stimulated or unstimulated plugs, nor in plugs incubated in either conditioned or fresh media. However, since SP70 synthesis was not observed in plugs incubated in media taken from the chamber between the plug and the electrode in these experiments, these control experiments did not make clear to what extent the agarose bridges isolated the specimens from electrode products.

In addition to inducing SP70 synthesis, electrode reaction products might change the rate of total protein synthesis. Heat-induced synthesis of SP70 is accompanied by changes in protein synthesis which depend on the length of exposure and on the temperature.

Total protein synthesis did not correlate with SP70 synthesis in the first

E1 E2 E3 C1 C2 C3    E1 E2 E3 C1 C2 C3

-140 kD

-SP70

A

B

Figure 3.2: Cartilage plugs labelled in a 6 hour electrical stimulation experiment at 10 Hz (gel fluorograph). No agarose plugs were used to separate the electrodes and the cartilage plugs.

(A) 3mm diameter plugs: electrically stimulated with  $9\text{mA}/\text{cm}^2$  (E1-E3) or matched unstimulated controls (C1-C3). These plugs were located in one middle position in the chamber (E1 and C1), in the position adjacent to E1 or C1, (E2 and C2) and at one end in the chamber (E3 and C3).

(B) 1/4 inch diameter plugs: electrically stimulated with  $2\text{mA}/\text{cm}^2$  (E1-E3) or matched unstimulated controls (C1-C3). The plugs were located (E1 and C1) at one end of the chamber, (E2 and C2) in the position adjacent to E2 or C2, and (E3 and C3) in one middle position in the chamber.

SP70 is apparent in A, lanes E2 and E3, and in B, lanes E1 and E2. These correspond to plugs located at the two ends of the stimulated chamber.

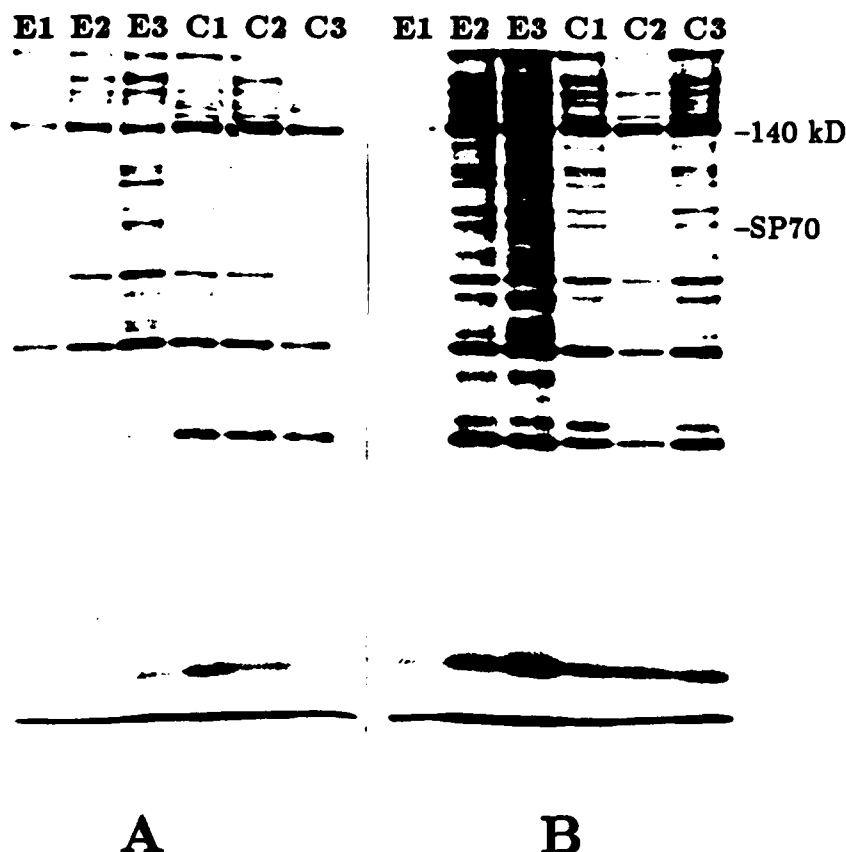


Figure 3.2: Cartilage plugs labelled in a 6 hour electrical stimulation experiment at 10 Hz (gel fluorograph). No agarose plugs were used to separate the electrodes and the cartilage plugs.

(A) 3mm diameter plugs: electrically stimulated with  $9\text{mA}/\text{cm}^2$  (E1-E3) or matched unstimulated controls (C1-C3). These plugs were located in one middle position in the chamber (E1 and C1), in the position adjacent to E1 or C1, (E2 and C2) and at one end in the chamber (E3 and C3).

(B) 1/4 inch diameter plugs: electrically stimulated with  $2\text{mA}/\text{cm}^2$  (E1-E3) or matched unstimulated controls (C1-C3). The plugs were located (E1 and C1) at one end of the chamber, (E2 and C2) in the position adjacent to E2 or C2, and (E3 and C3) in one middle position in the chamber.

SP70 is apparent in A, lanes E2 and E3, and in B, lanes E1 and E2. These correspond to plugs located at the two ends of the stimulated chamber.



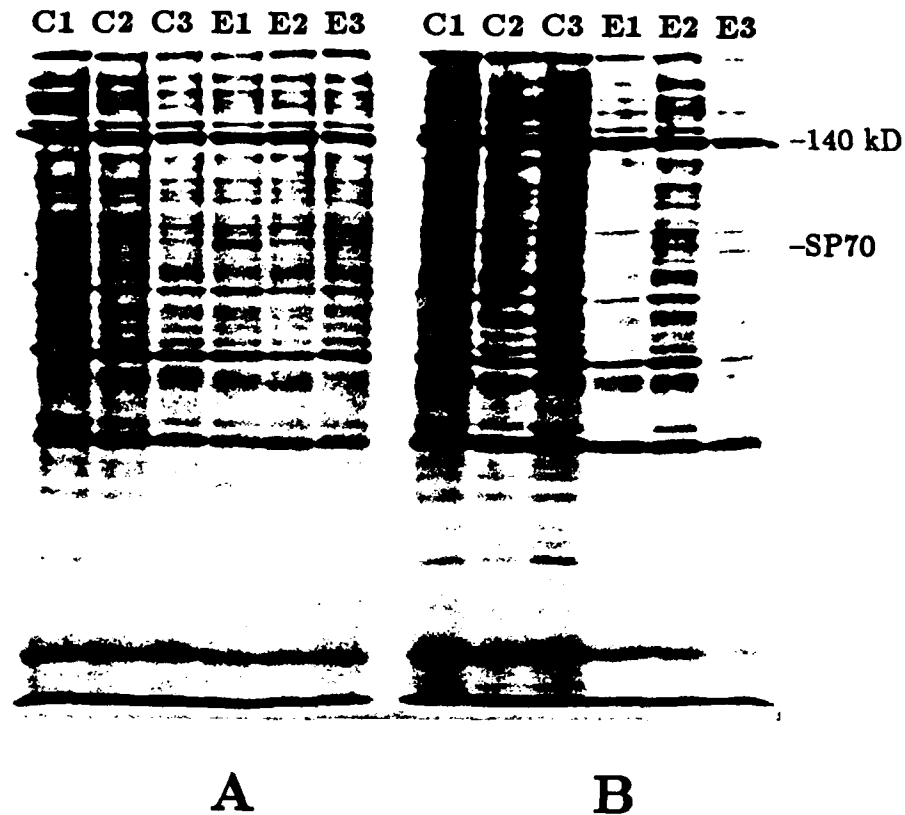


Figure 3.3: Cartilage plugs incubated either in media taken from the chamber adjacent to each cartilage holder position after an electrical experiment or in fresh media (fluorograph) (A) 3mm diameter plugs, incubated in media from the chamber (E1-E3) or in fresh media (C1-C3). The media was taken from (E1) between the electrode and first plug, (E2) between the first and second plug, and (E3) between the second and third plug.  
(B) 1/4 inch plugs, incubated in media from the chamber (E1-E3) or in fresh media (C1-C3). The media was taken from (E1) between the third and second plug, (E2) between the first and second plug, and (E3) between the first plug and the electrode. SP70 is apparent in A, lane E1 and B, lane E3. These correspond to plugs incubated in media taken from each end of the chamber.

C1 C2 C3 E1 E2 E3    C1 C2 C3 E1 E2 E3

-140 kD

-SP70

A

B

Figure 3.3: Cartilage plugs incubated either in media taken from the chamber adjacent to each cartilage holder position after an electrical experiment or in fresh media (fluorograph) (A) 3mm diameter plugs, incubated in media from the chamber (E1-E3) or in fresh media (C1-C3). The media was taken from (E1) between the electrode and first plug, (E2) between the first and second plug, and (E3) between the second and third plug.

(B) 1/4 inch plugs, incubated in media from the chamber (E1-E3) or in fresh media (C1-C3). The media was taken from (E1) between the third and second plug, (E2) between the first and second plug, and (E3) between the first plug and the electrode. SP70 is apparent in A, lane E1 and B, lane E3. These correspond to plugs incubated in media taken from each end of the chamber.

electrical stimulation and conditioned media experiment. That is, total protein synthesis in plugs in which SP70 had been induced by electrode reaction products did not appear to be much different relative to plugs in which SP70 was not induced. In addition, the mean of  $^{35}\text{S}$ -methionine incorporation by electrically stimulated plugs was not significantly different relative to control plugs, but sample to sample variation was large. Total  $^{35}\text{S}$ -methionine incorporation in plugs incubated in either media from the stimulated or unstimulated chamber was low relative to matched controls in fresh media (Table 3.1).

In one experiment in which agarose plugs were used no increase in SDS-soluble  $^{35}\text{S}$ -methionine incorporation was observed. In the other, which was at higher current density, SDS-soluble  $^{35}\text{S}$ -methionine incorporation was greater in electrically stimulated plugs relative to their matched controls. In this experiment, incorporation in fresh plugs incubated in media from the end position (between electrode and agarose plug) of the stimulated chamber was lower than in matched plugs incubated in fresh media ( $p < 0.05$ ). However, SDS-soluble incorporation in plugs incubated in conditioned media positions adjacent to the cartilage holders was not significantly different relative to their matched controls.

In summary, total SDS-soluble protein synthesis was lower in specimens incubated in media from the end position, between the agarose plug and electrode, and approximately equal relative to controls in specimens incubated in media from all other positions. These results suggest that the agarose plug isolates the electrode. Furthermore, no SP70 has been induced in electrically stimulated plugs or in plugs incubated in media from the chamber in subsequent experiments using agarose bridges.

### 3.2.3 Temperature Measurements

The temperature of the media in the stimulation and control chambers was measured during some electrical experiments using current densities less than  $4 \text{ mA/cm}^2$

Experimental conditions	Stress response	cpm in SDS-soluble extract
10 Hs, 6 hrs (no agar bridge) 2 mA/cm <sup>2</sup> (1/4 in. plugs) 9 mA/cm <sup>2</sup> (3 mm plugs)	SP70 was induced in 2 plugs at each end of the stimulated chamber	0.91 ± 0.38 (1/4 in.; n = 3) 1.03 ± 1.00 (3 mm; n=3) <u>electrically stimulated</u> unstimulated
incubation in media from stimulated chamber (after electrical experiment)	SP70 was induced in 2 plugs: plugs incubated in 1 ml of media from the end positions of the chamber	0.81 ± 0.24 (all plugs) 0.84 ± 0.39 (ends) <u>in stimulated media</u> in fresh media
incubation in media from unstimulated chamber	none	0.77 ± 0.12 (all) 0.53 (1 end) <u>in unstimulated media</u> in fresh media
10 Hs, 12 hrs (with agar bridge) 6 mA/cm <sup>2</sup> (1/4 in. plugs) 27 mA/cm <sup>2</sup> (3 mm plugs)	none	1.40 ± 0.86 (1/4 in.; n = 3) (no end) 1.16 (n = 1) (end plug) <u>electrically stimulated</u> unstimulated
incubation in media from stimulated chamber (after electrical experiment)	none	0.393 ± .10 (electrode-agar) 0.836 ± 0.02 (agar-1st plug) 0.93 ± .47 (middle plugs:2-5) <u>in stimulated media</u> in fresh media
incubation in media from unstimulated chamber (after electrical experiment)	none	0.70 ± .10 (electrode-agar) 1.04 ± 0.04 (agar-1st plug) 0.95 ± .24 (middle plugs:2-5) <u>in unstimulated media</u> in fresh media
10 Hs, 12 hrs (with agar bridge) 30 mA/cm <sup>2</sup> (3 mm plugs)	none	2.53 ± 1.00 (n = 6) <u>electrically stimulated</u> unstimulated
incubation in media from stimulated chamber (after electrical experiment)	none	0.430 ± 0.04 (electrode-agar) 1.06 ± 0.14 (agar-1st plug) 1.10 ± .12 (middle plugs:235) (n=3) <u>in stimulated media</u> in fresh media

Table 3.1: Conditioned media experiments: SDS-soluble counts

for 1/4 inch plugs, and 20 mA/cm<sup>2</sup> for 3 mm diameter plugs. The temperature was measured by means of thermistors (Thermometrics, series p60) inserted into the cartilage holders, such that the cartilage specimen could be inserted adjacent to the thermistor (Fig. 3.1). The thermistors were inserted into the holder and the thermistor and bridge circuit system was calibrated prior to autoclaving the thermistors. Cartilage samples were loaded at room temperature under a hood, so that there was a temperature transient associated with the rise to incubator temperature. The temperature measured in the media adjacent to a cartilage plug rose from room temperature to within 0.5°C of 37°C within 2 hours when placed in the incubator. Fig. 3.4, for example shows the temperature monitored for a current density of 8.5 mA/cm<sup>2</sup>, which was turned on a few minutes after the chamber was placed in the incubator. Temperature in the experimental and control chambers tracked closely. Final temperatures did not differ by more than  $\pm 0.2^\circ\text{C}$  for up to 20 mA/cm<sup>2</sup> in 6 hours, indicating that effects seen are not likely to be due to ohmic heating (Table 3.2).

In a separate experiment, the temperature was measured experimental relative to control chambers during stimulation at the maximum current density (30 mA/cm<sup>2</sup>) after 12 hours. The chamber was filled with media, with holders in place, but without cartilage plugs in the holders and stimulated for 12 hours at 30 mA/cm<sup>2</sup>. After 12 hours, the bridge circuits for the experimental and control thermistors were zeroed. The current was then turned off in the electrical stimulation chamber, and the temperature recorded for 3 hours. The experiment was repeated using a stimulation current density of 20 mA/cm<sup>2</sup>. Thermistors were calibrated immediately following the experiment. The difference in temperature in the stimulated chamber with 30 mA/cm<sup>2</sup>, relative to the temperature with the current off was less than 0.15°C (Fig. 3.5 A). Relative to the control chamber it was less than 0.2°C. At 20 mA/cm<sup>2</sup>, the relative differences were as small as variation in incubator temperature over the same time period (Fig. 3.5 B).

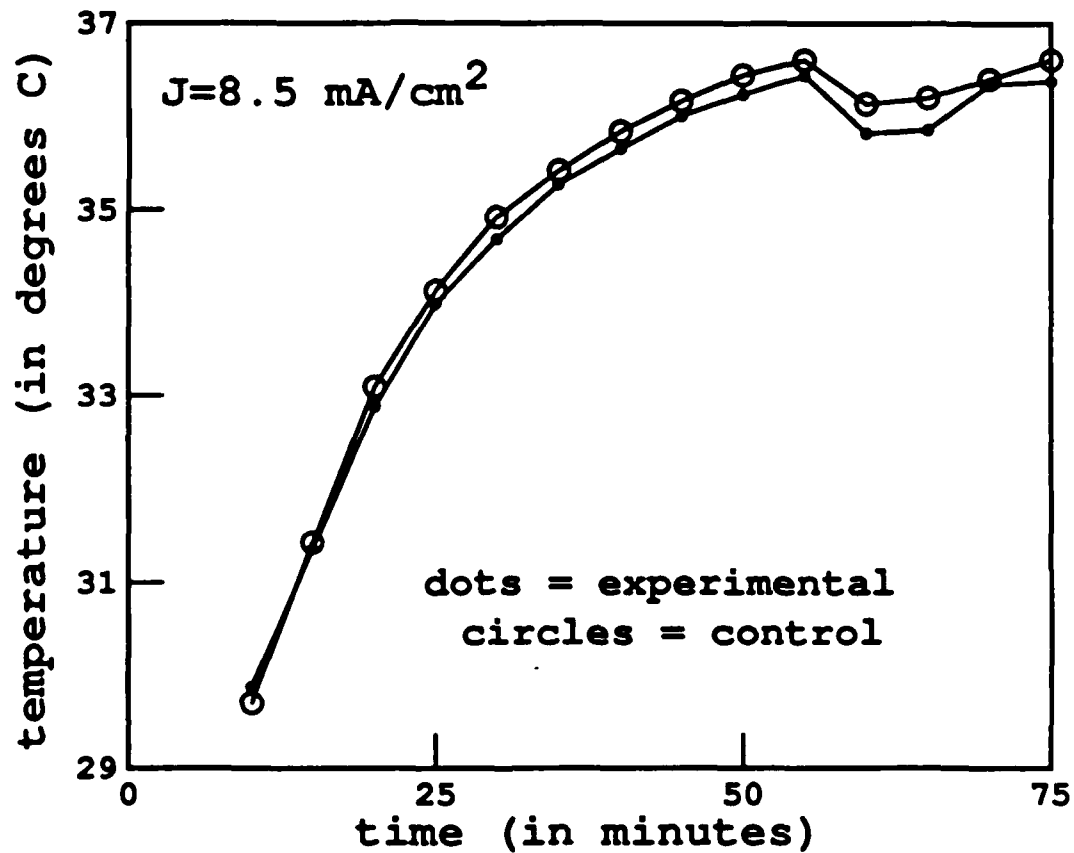


Figure 3.4: Temperature measured by thermistors in the media adjacent to the middle plugs in the stimulated and control chamber, as a function of the time after the chamber was placed in the incubator.

Current density (mA/cm <sup>2</sup> )		Final temperature in stimulation cham- ber, after 6 hour stim- ulation (°C)	Final temperature in control chamber (°C)
1/4 inch plugs	3 mm plugs		
1	4.5	37.1	36.9
2	9	37.2	37.3
3.5	15.7	37.5	37.6
4	18	37.5	37.3

Table 3.2: The final temperature measured after 6 hours of electrical stimulation, (at the current density given in the experimental chamber, and with no current in the control chamber) in control and experimental chambers.

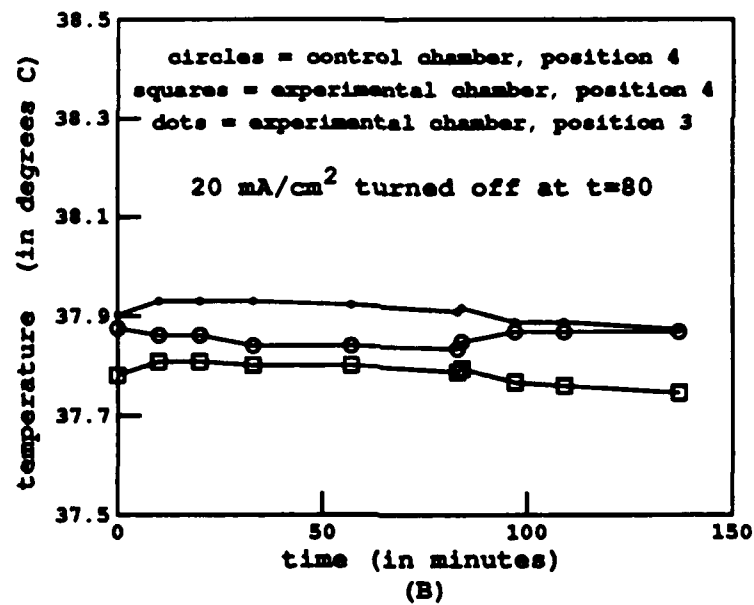
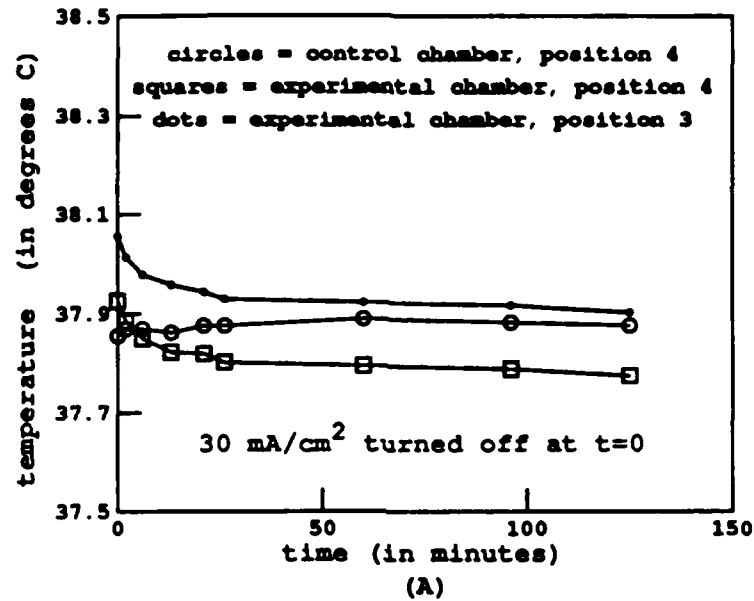


Figure 3.5: Temperature measured by thermistors in the cartilage holders in the experimental and control chambers.

(A) Measured following stimulation at 30 mA/cm<sup>2</sup>. The current was turned off at t=0.

(B) Measured during and following stimulation at 20 mA/cm<sup>2</sup>. The current was turned off at t=80.



### 3.3 Electrical Stimulation Experiments

Electrical stimulation experiments were done over a range of frequencies and current densities. Initial pilot studies (Appendix C.3) suggested that current densities below 10 mA/cm<sup>2</sup> had no effect on protein synthesis. An increase in total protein synthesis was found in an experiment at 30 mA/cm<sup>2</sup>. Therefore, the stimulation amplitudes were chosen to span the range between 10 and 30 mA/cm<sup>2</sup>. The frequency range was chosen to span the range 1 Hz to 10 kHz, as discussed previously. Experiments were done at 1, 10, 100 Hz, and 1 and 10 kHz. Following results from initial experiments which suggested that field induced effects on total protein synthesis might depend on core position on the joint surface, all cartilage plugs obtained from a single facet of the joint surface were used in a single stimulation protocol.

Table 3.3 summarizes the amplitude and frequency of stimulation, and the positions on the joint surface from which the plugs were taken for all experiments. The joint positions are labelled according to the convention in Fig. 2.1 (Chapter 2).

#### Effect of Serum Concentration

To examine the possibility that the effects of electrical stimulation were dependent on culture and labelling conditions, plugs were cultured and then labelled in media supplemented with either 0.1 %NS (DMEM-N) or with 10 %FBS (DMEM-F) and stimulated simultaneously with the same frequency and current density. In comparing the effects of applied fields in the two serum concentrations, the right and left joints from the same cow were matched, as described below. Right and left joints have similar baseline synthesis, when cultured under the same conditions (as shown by control studies described in Appendix B.7). The response to electric fields was similarly assumed to be independent of right or left joint, when cultured under the same conditions, as shown in Section 3.3.1.

Plugs were cultured for 5 days in DMEM-F with daily media changes or in cultured in DMEM-N with media changes every two days, as usual.

J (mA/cm <sup>2</sup> ) frequency	< 10	10	10-20	20	20-30	30
1 Hz		135 LM		3-5 LL 3-5 RL		1-5 LM
		246 LM		1-4 LL 1345 RL		1234 RM
10 Hz		135 RM			4RL	1LM 1LL 3RM 6LM
100 Hz		246 RM	246 LM 246 RM* (J=12.5)	135 LL* 135 RL	2LM 5LM 1RM (J=24)	5LL 5LM 6LM
				1LL 4LL 4RL 4LM 4RM 6RM		135 RM* 135 LM
1 kHz	2RM 3RM 4LM (J=8)	1-5 RL		1-5 LL		
10 kHz		1-6 RM		1-6 LM		135 RL 135 LL

Table 3.3: Electrical stimulation experiments, listed in terms of amplitude and frequency. The core positions of the plugs used in each experiment are given using the notation of Fig. 2.1 (RL=right lateral facet, RM = right medial facet, LL = left lateral facet and LM = left medial facet. Position 1 is the most proximal position, position 5 or 6 is the most distal position on the joint surface.)

In one set of experiments, six experimental and six control plugs (core positions 2 4 6LM) were stimulated and labelled in DMEM-N and six experimental and six control plugs (core positions 2 4 and 6RM) were stimulated and labelled in media containing 10% FBS using a current density of  $12.5 \text{ mA/cm}^2$  at 100 Hz. One experimental and control chamber pair was filled with media containing 0.1%NS; and the other experimental and control chamber pair was filled with media containing 10%FBS. The electrodes of the experimental chambers were connected to two channels of the current source. The assumption that cartilage plugs from the right and left joint were biosynthetically equivalent when cultured under the same conditions was checked, for this particular cow, by stimulating (using  $20 \text{ mA/cm}^2$  at 1 Hz) and labelling plugs from the both right and left facets (positions 3 4 5RL and 3 4 5LL) that had been maintained in 0.1%NS.

In a second set of experiments, plugs were stimulated at 100 Hz for 12 hours at  $20 \text{ mA/cm}^2$  (plug positions 1 3 and 5 LL in DMEM-F and 1 3 and 5 RL in DMEM-N) and at  $30 \text{ mA/cm}^2$  (plug positions 1 3 and 5 RM in DMEM-F, and 1 3 and 5 LM in DMEM-N). A single experimental and control chamber was used. One half of the experimental and of the control chamber was filled with 0.1% NS, and the other half was filled with media containing 10% FBS separated by two 1.5 cm agarose plugs. As before, the assumption that plugs from the right and left joints were biosynthetically equivalent was checked by stimulating (100 Hz at  $20 \text{ mA/cm}^2$ ) and labelling plugs from the both right and left facets (4RL 4LL, 4RM and 4LM), that had been maintained in 0.1%NS.

### **3.3.1 Results**

#### **No Stress Response was Induced by Electric Fields**

No SP70 or any other heat shock protein was detected by SDS-PAGE for plugs stimulated at any current density. In all cases, SP70 was induced in control cartilage plugs obtained from the same cows used in the same electrical experiments by

incubation at elevated temperatures (41-43°C).

### **Total Protein Synthesis was Increased by Electric Fields**

Although SP70 was not induced, there was an increase in total protein synthesis for current densities greater than 10 mA/cm<sup>2</sup>. The experimental results are summarized graphically in Fig. 3.6 to 3.9. SDS soluble radioactivity normalized to wet weight is plotted as a ratio of experimental plugs to their matched control. Within a single frequency (such as 100 Hz) incorporation into the SDS extract, the papain digest, and their sum—the total incorporation, appears to increase with current density (Fig. 3.6 to 3.8). The ratio of incorporation into the SDS-soluble fraction to incorporation in the papain digest, has a corresponding decrease, except at 10 kHz. This same data is plotted in Fig 3.10 to 3.12. as a function of frequency at a given current density (10, 20 or 30 mA/cm<sup>2</sup>). There is not a clear dependence on frequency up to 10 kHz, although there is a peak in response near 100 Hz - 1 kHz at 20 and 30 mA/cm<sup>2</sup> suggestive of a maximum in this frequency range.

Average experimental to control ratios for total incorporation were greater than 1 for all experiments but 2, both using 10 mA/cm<sup>2</sup> (at 10 and 100 Hz). Using the students t test, only the increases at 30 mA/cm<sup>2</sup>, 100 Hz and at 20 and 30 mA/cm<sup>2</sup>, 10 kHz are significant ( $p < .05$ ). The decrease in the ratio of SDS-soluble to papain digest radioactivity is significant for these same experiments.

### **Statistical Tests on Increased Total Incorporation**

Statistical results have been reported throughout in terms of the t test (with correction using the Bonferroni inequality when more than 2 groups were compared). Additional statistical tests were used to examine the increase in total protein synthesis.

To test the hypothesis that total incorporation increased with current density, linear regressions of the increase in total incorporation vs current density were

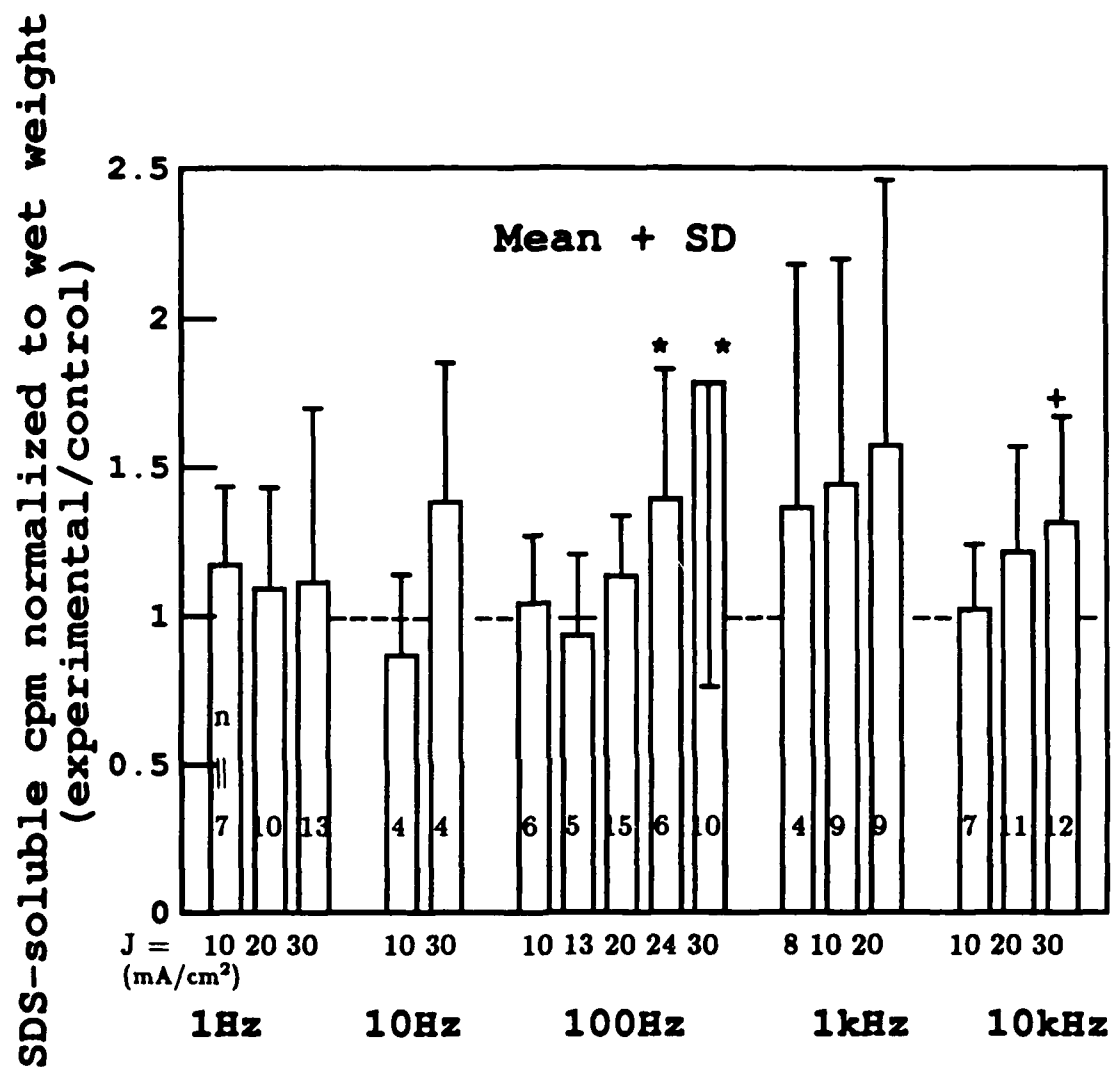


Figure 3.6: Summary of SDS-soluble counts (cpm), as a function of current density and frequency ( \*  $p < 0.05$ , +  $p < 0.002$ ).

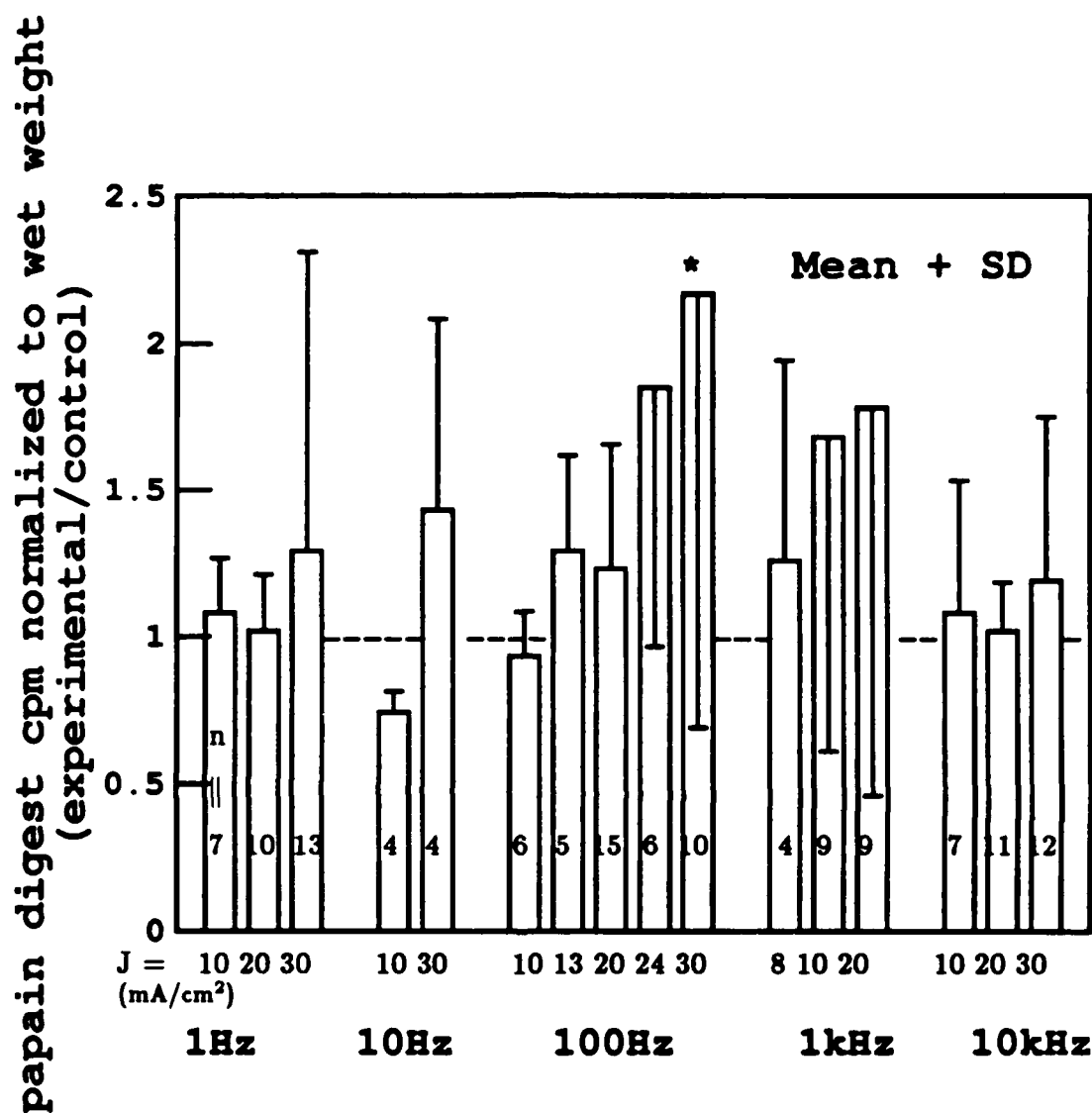


Figure 3.7: Summary of papain digest counts, as a function of current density and frequency ( \*  $p < 0.05$ , +  $p < 0.01$ ).

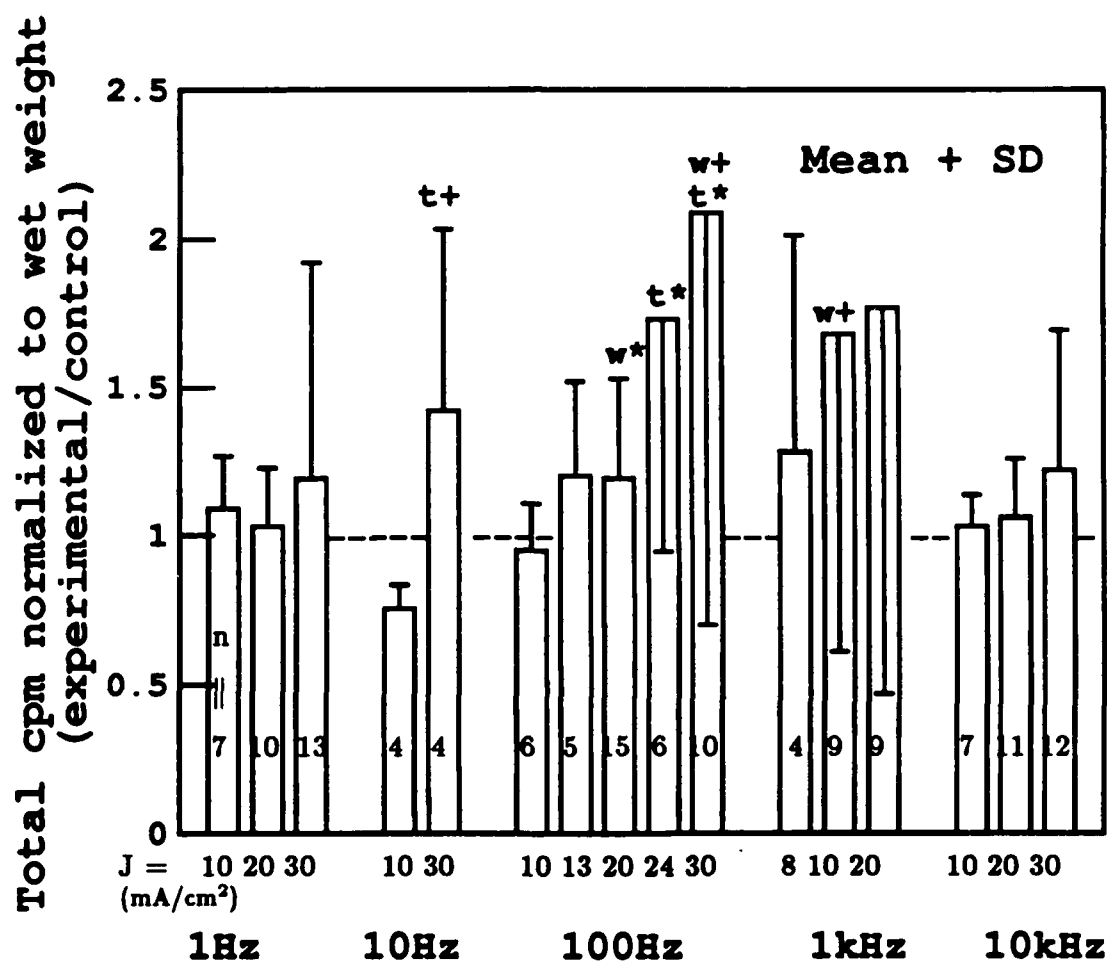


Figure 3.8: Summary of total counts (the sum of SDS-soluble and papain digest extracts) as a function of current density and frequency (t\*, w\* p<0.05, t+, w+ p<0.02, using the t test and Wilcoxon test, respectively).

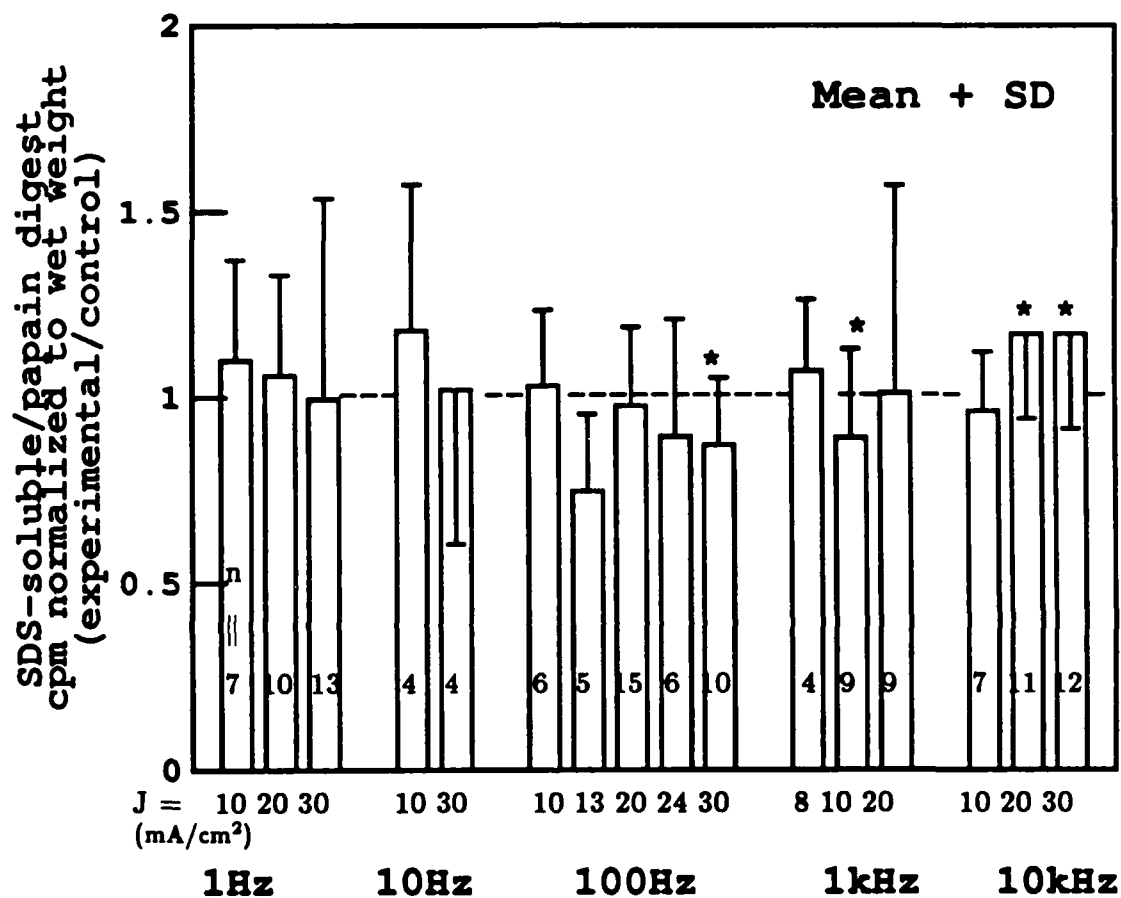


Figure 3.9: Summary of the ratio of SDS-soluble to papain digest counts, as a function of current density and frequency (\*  $p < 0.05$ ).



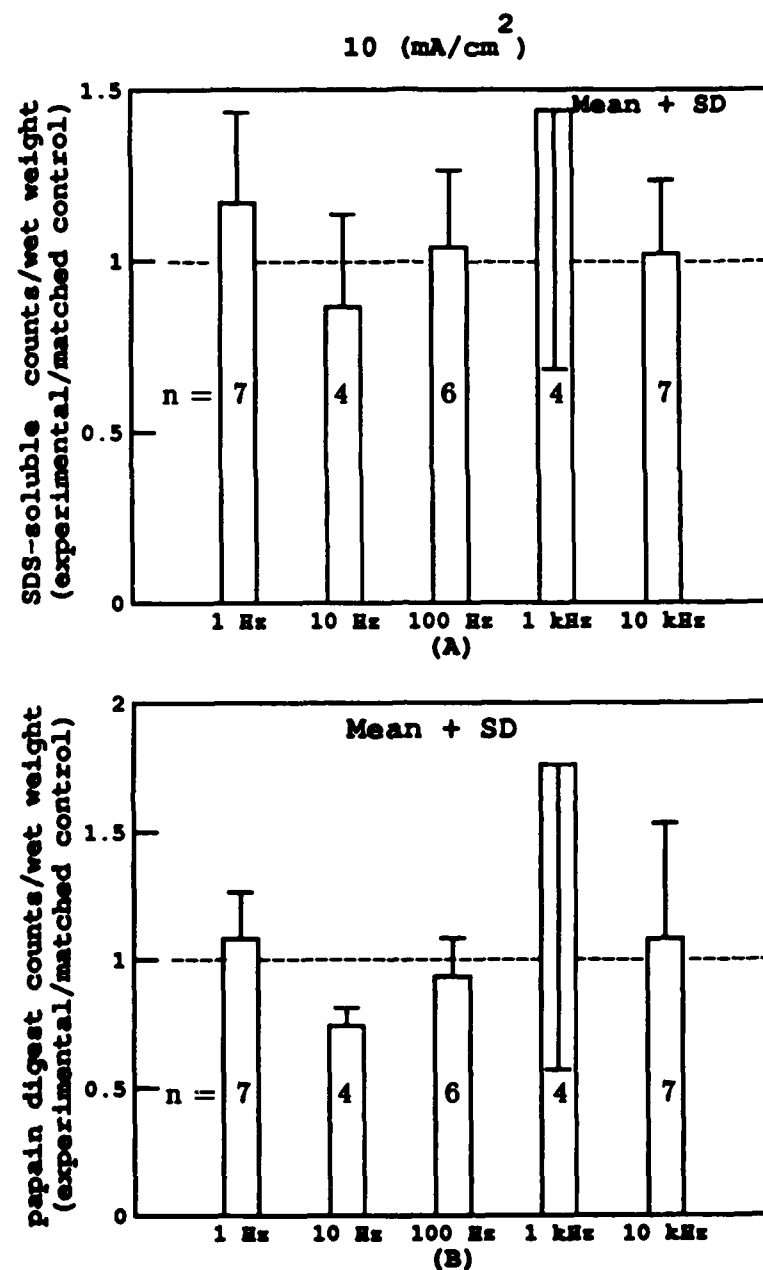


Figure 3.10: Total radioactive counts (cpm) in the (A) SDS-soluble extract or (B) papain digest, normalized to wet weight are plotted as the ratio of experimental to control specimens as a function of frequency at  $10 \text{ mA/cm}^2$ .

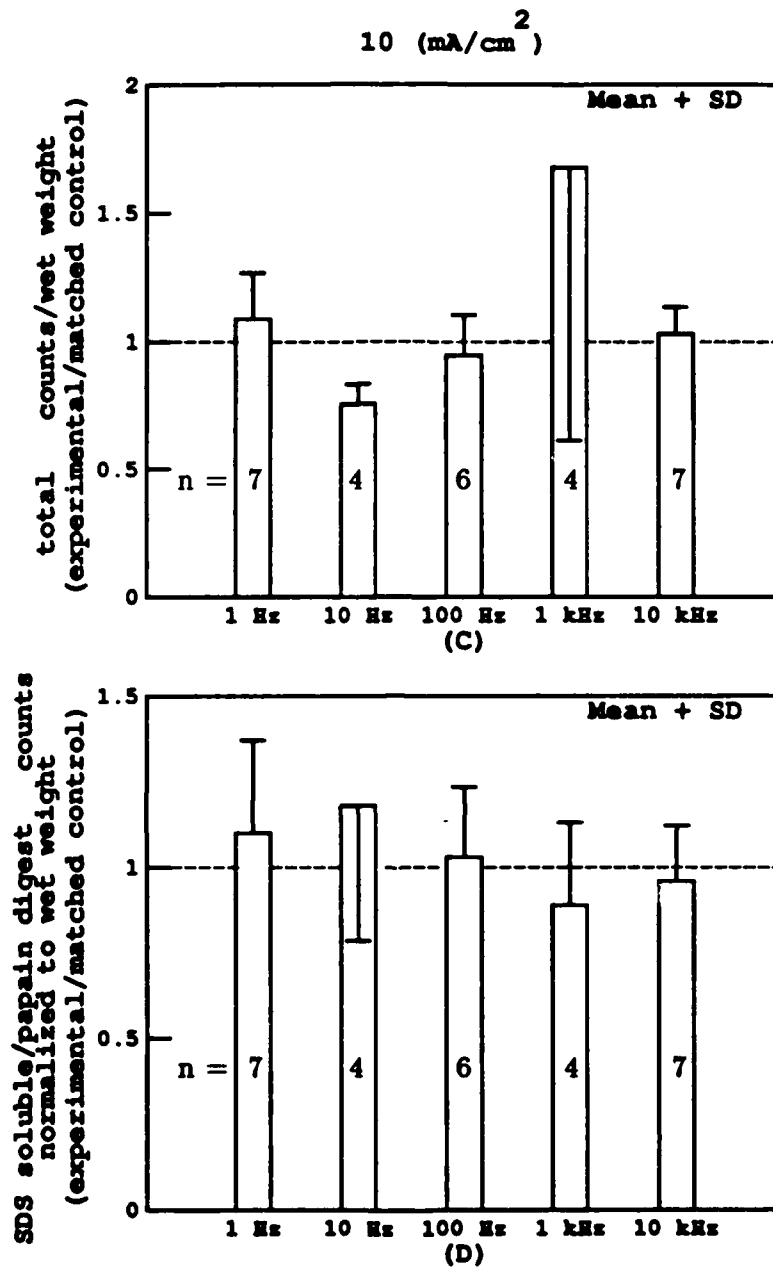
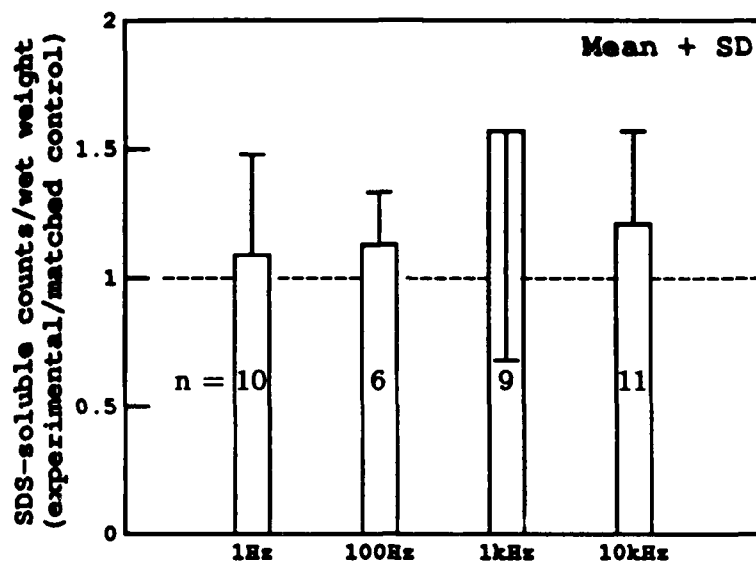
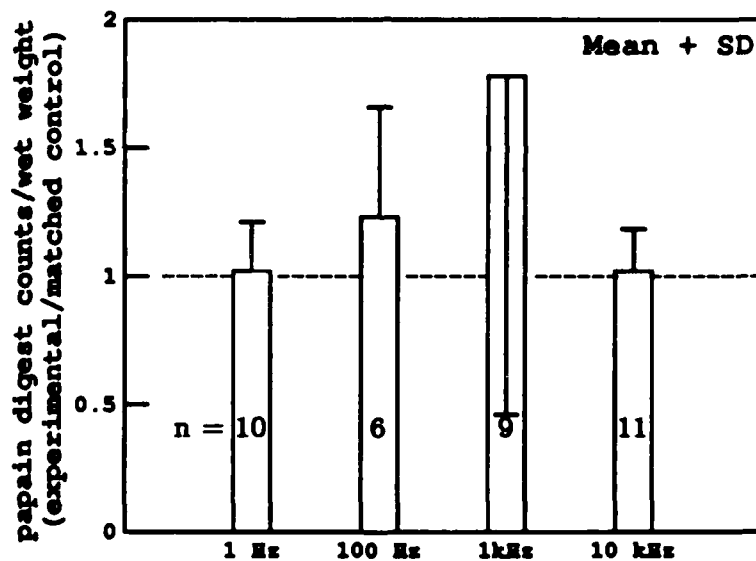


Figure 3.10: (C) Total radioactive counts in SDS-soluble and papain digest extracts or (D) the ratio of radioactive counts in the SDS-soluble extract to the papain digest. Radioactivity (cpm) is normalized to wet weight and plotted as the ratio of experimental to control specimens as a function of frequency at  $10 \text{ mA/cm}^2$ .

20 (mA/cm<sup>2</sup>)



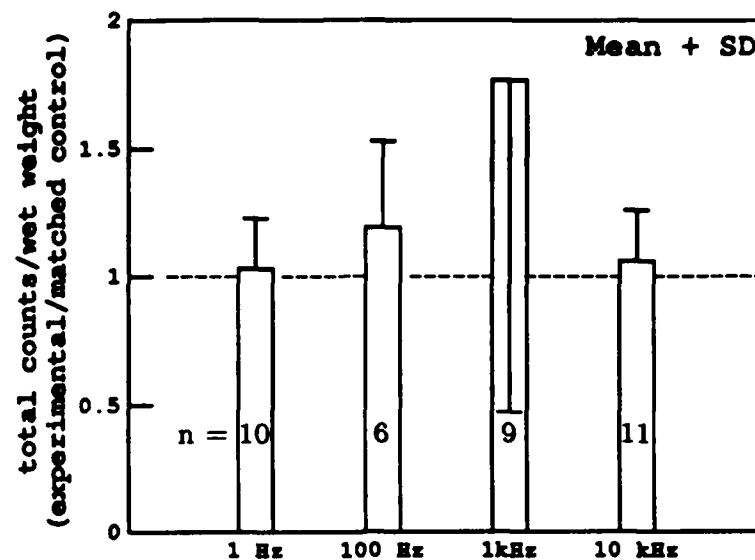
(A)



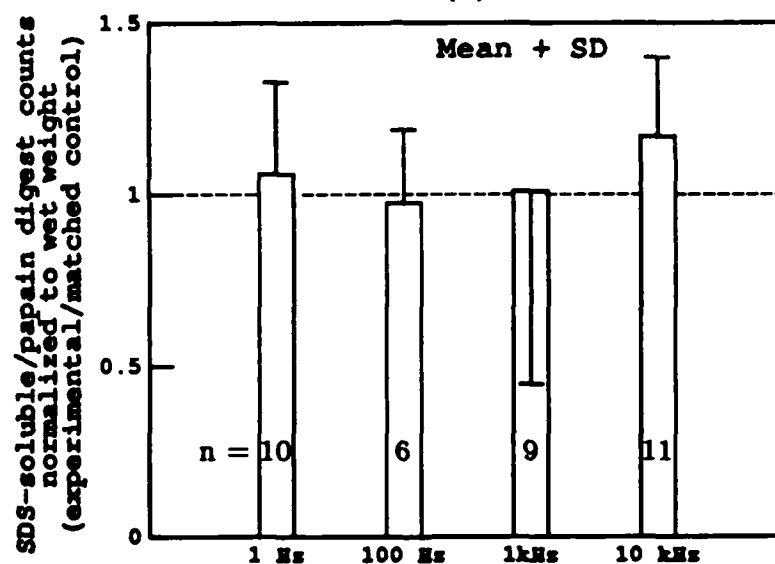
(B)

Figure 3.11: Total radioactive counts (cpm) in the (A) SDS-soluble extract or (B) papain digest, normalized to wet weight are plotted as the ratio of experimental to control specimens as a function of frequency at 20 mA/cm<sup>2</sup>.

-160-  
20 (mA/cm<sup>2</sup>)



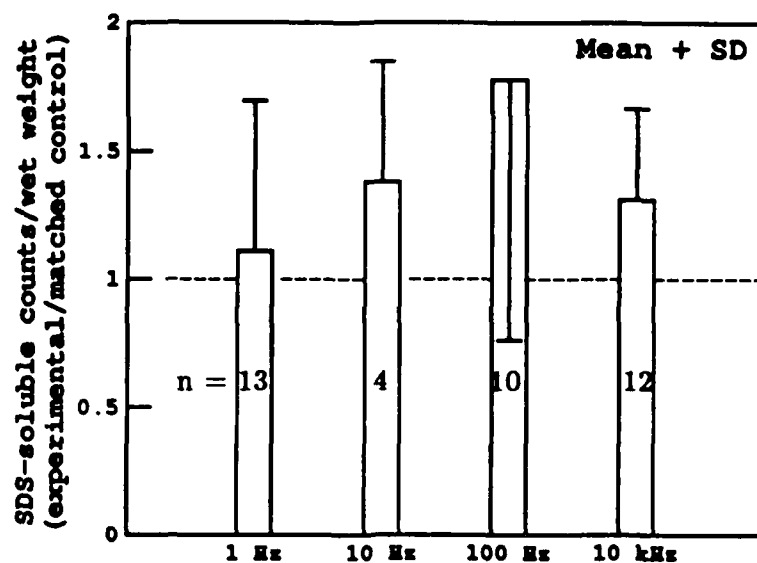
(C)



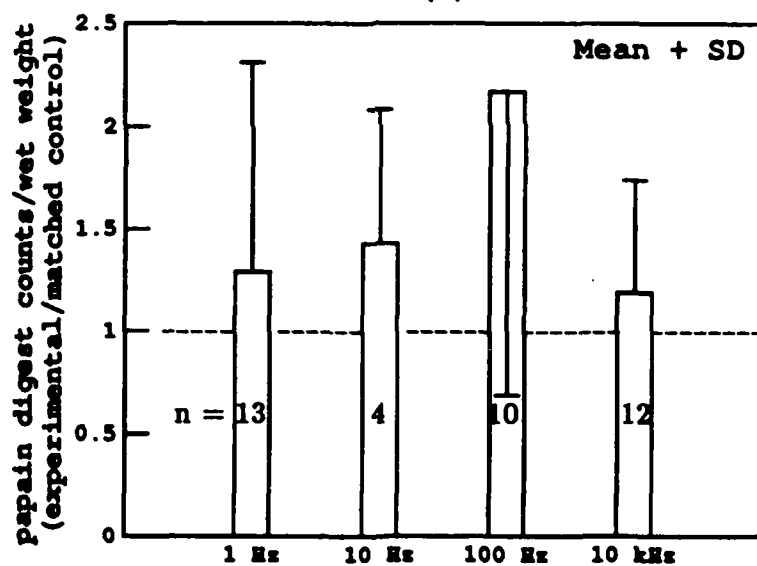
(D)

Figure 3.11: (C) Total radioactive counts in SDS-soluble and papain digest extracts or (D) the ratio of radioactive counts in the SDS-soluble extract to the papain digest. Radioactivity (cpm) is normalized to wet weight and plotted as the ratio of experimental to control specimens as a function of frequency at 20 mA/cm<sup>2</sup>.

30 (mA/cm<sup>2</sup>)



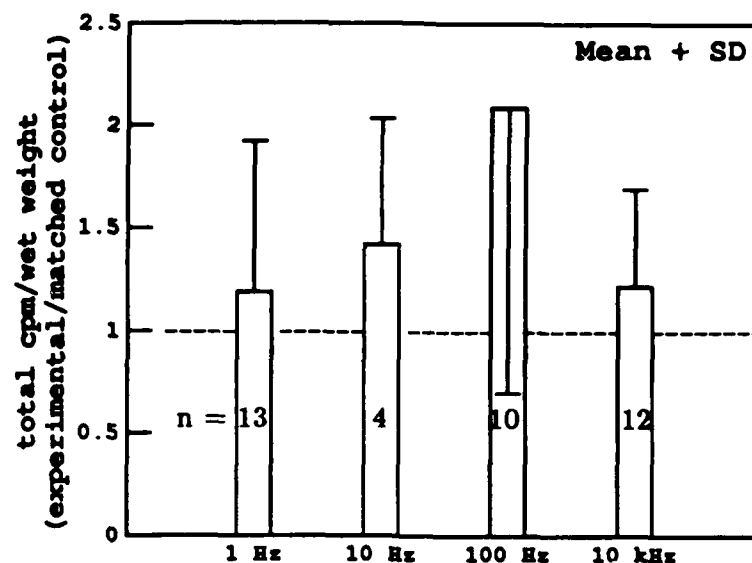
(A)



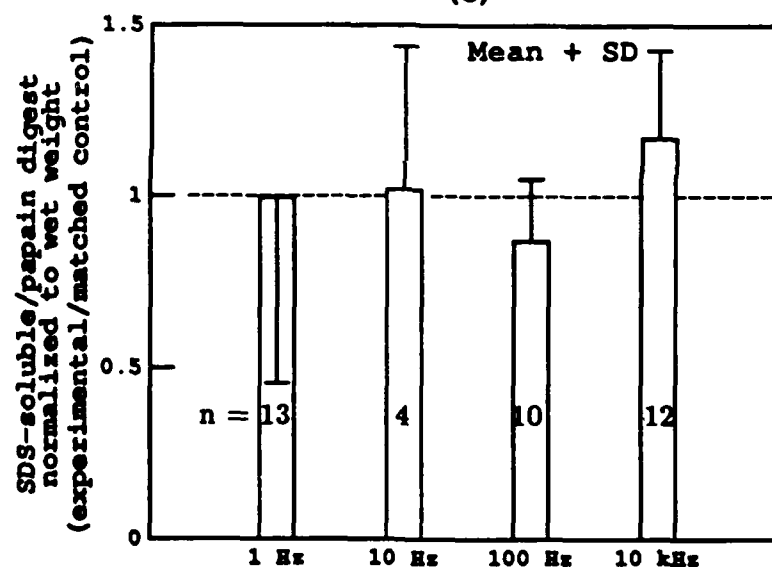
(B)

Figure 3.12: Total radioactive counts (cpm) in the (A) SDS-soluble extract or (B) papain digest, normalized to wet weight are plotted as the ratio of experimental to control specimens as a function of frequency at 30 mA/cm<sup>2</sup>.

30 (mA/cm<sup>2</sup>)



(C)



(D)

Figure 3.12: (C) Total radioactive counts in SDS-soluble and papain digest extracts or (D) the ratio of radioactive counts in the SDS-soluble extract to the papain digest. Radioactivity (cpm) is normalized to wet weight and plotted as the ratio of experimental to control specimens as a function of frequency at 30 mA/cm<sup>2</sup>.

**Methionine Incorporation vs  
current density at f=100 Hz**

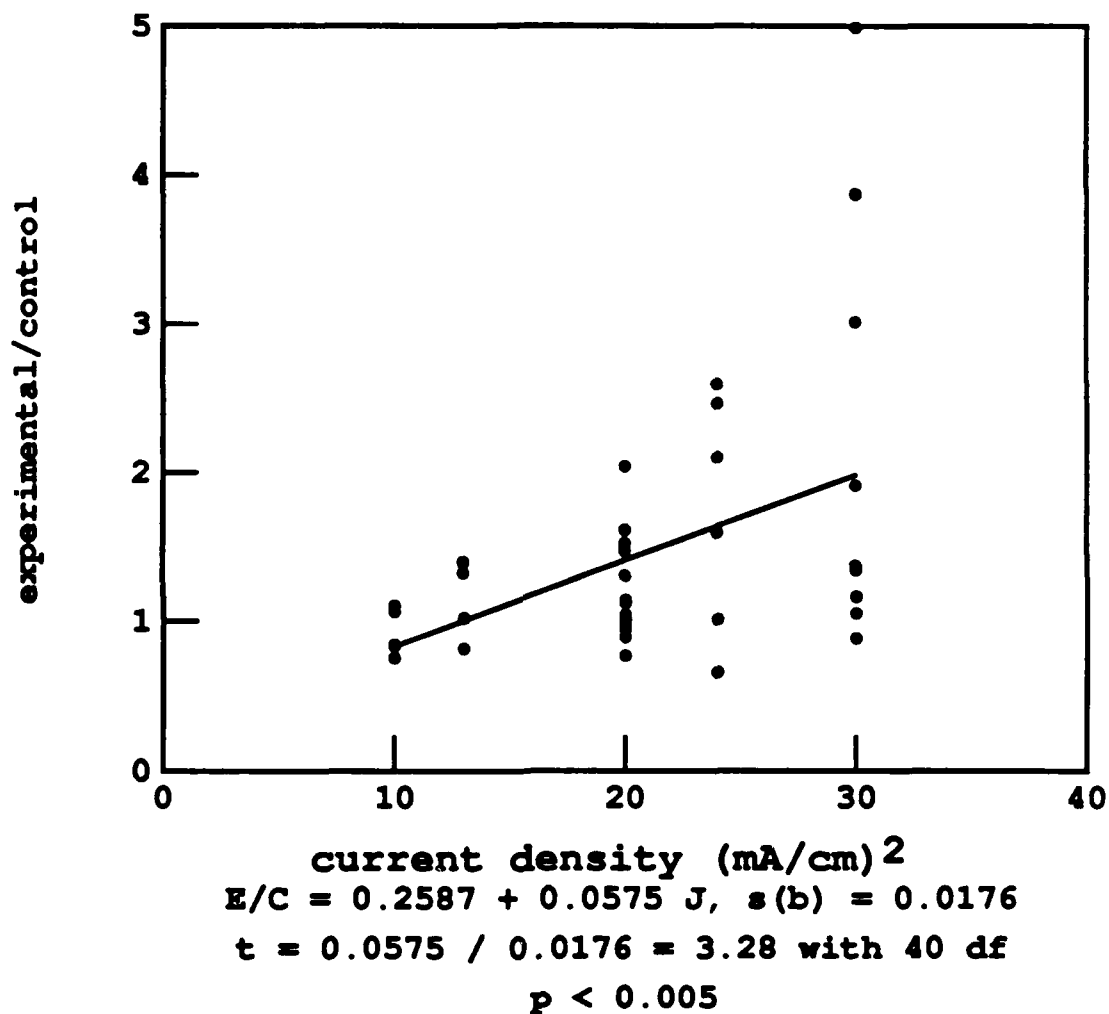


Figure 3.13: Linear regression of total incorporation vs. current density at 100 Hz.

within done each frequency. Total incorporation was shown to increase with current density at 100 Hz ( $p < 0.005$ , Fig. 3.13). The increase with current density was not statistically significant by linear regression at any other frequency.

Qualitatively it often appeared that the distribution of the experimental/control ratios for a given experiment was not described by a normal distribution (see e.g., Fig. 3.13). Consequently, non-parametric methods were also used to test the significance of the increase in total incorporation. The Wilcoxon test was used to find the significance of the increase in incorporation for each current density and frequency. In addition, data from all experiments at a single current density were pooled to examine whether these current densities produced a significant increase in total incorporation independent of frequency using the Wilcoxon test. Finally, to examine the dependence of total incorporation on amplitude within a single frequency, the Kruskal Wallis test was used.

The Wilcoxon test applied to the data at each current density and frequency showed a significant increase in incorporation in electrically stimulated plugs for current densities of 10 mA/cm<sup>2</sup> at 1 kHz, and at 20 mA/cm<sup>2</sup> and 30 mA/cm<sup>2</sup> at 100 Hz.

In order to examine the response as a function of current density over all experiments, data were pooled as a function of amplitude independent of frequency. Pooling the data in this manner assumes that the response to stimulation is essentially independent of frequency. Hence, this pooling tests the hypothesis that the significance of the trend could be hidden by dividing the population into many small subgroups each having a small "n". The Wilcoxon test applied to pooled data for experiments at 20 mA/cm<sup>2</sup> (frequencies of 1 and 100 Hz and 1 and 10 kHz), showed a significant increase in total incorporation ( $p < 0.02$ ). For pooled data at 30 mA/cm<sup>2</sup>, the significance level was  $p = .053$ .

The Kruskal Wallis test was used to examine differences in incorporation with current density within each frequency. At 100 Hz, the populations were found



to be statistically different ( $p < 0.005$ ). At no other frequency were the differences significant.

### **The Field Induced Increase in Incorporation Depended on Plug Position**

The large standard deviations in the experimental/control ratios and in the control population appeared to be associated with biosynthetic variation over the joint surface. That is, the response depended on the core position on the joint surface from which the plugs were taken. In general, the largest standard deviations corresponded to experiments showing the largest increase in incorporation in electrically stimulated plugs. The positional dependence of the response can be best seen by focusing on the response of pairs of plugs from a single joint position in the experiments with large increases and large standard deviations. Figs. 3.14 and 3.15, for example, represent the same data from the experiments at 24 and 30 mA/cm<sup>2</sup> at 100 Hz (in Figs. 3.6 to 3.9), but replotted for averages of 2 pairs of experimental/matched controls of plugs from each of three core positions (proximal—position 1—to distal—position 5 or 6). The differences in electrical response between plugs from the same core position are smaller than the differences between plugs from different cores.

Based on these results, electrical stimulation experiments were done plugs from a single joint facet to explore the positional variation. Qualitatively, it appeared in these experiments, that on average the differences in response for two pairs of plugs from a single core were smaller than differences between pairs of plugs from different cores. Furthermore, the experimental to control ratios within an experiment tended to have a recurring pattern with position on the joint surface, independent of stimulation frequency or amplitude. The increase in incorporation in response to electrical stimulation tended to be greater in plugs from central joint positions (i.e. 2-5 on the medial facet, and 2-4 on the lateral facet), and least for end positions. Fig. 3.16 shows an example of this pattern for an experiment at

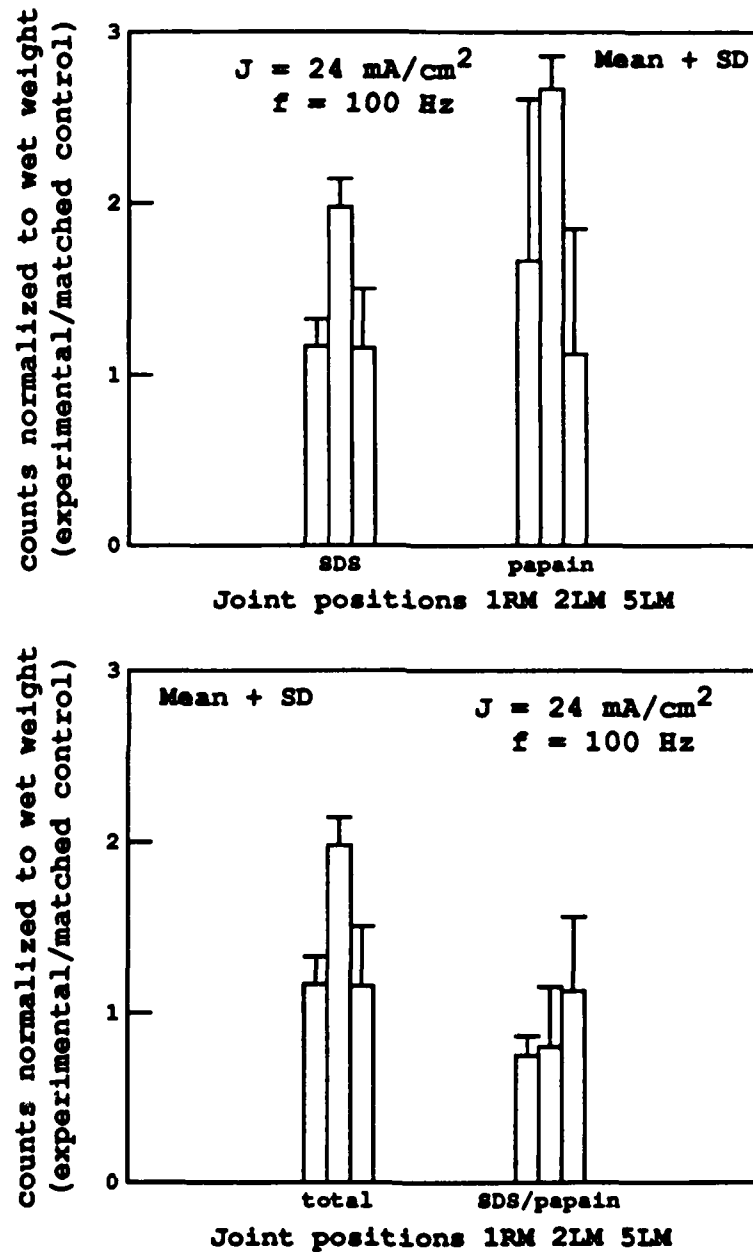


Figure 3.14: Electrical response by core position ( $f = 100 \text{ Hz}$ ,  $J = 24 \text{ mA/cm}^2$ ). Experimental/control ratios (cpm normalized to wet weight) are plotted for averages of 2 pairs of matched plugs from the same core.

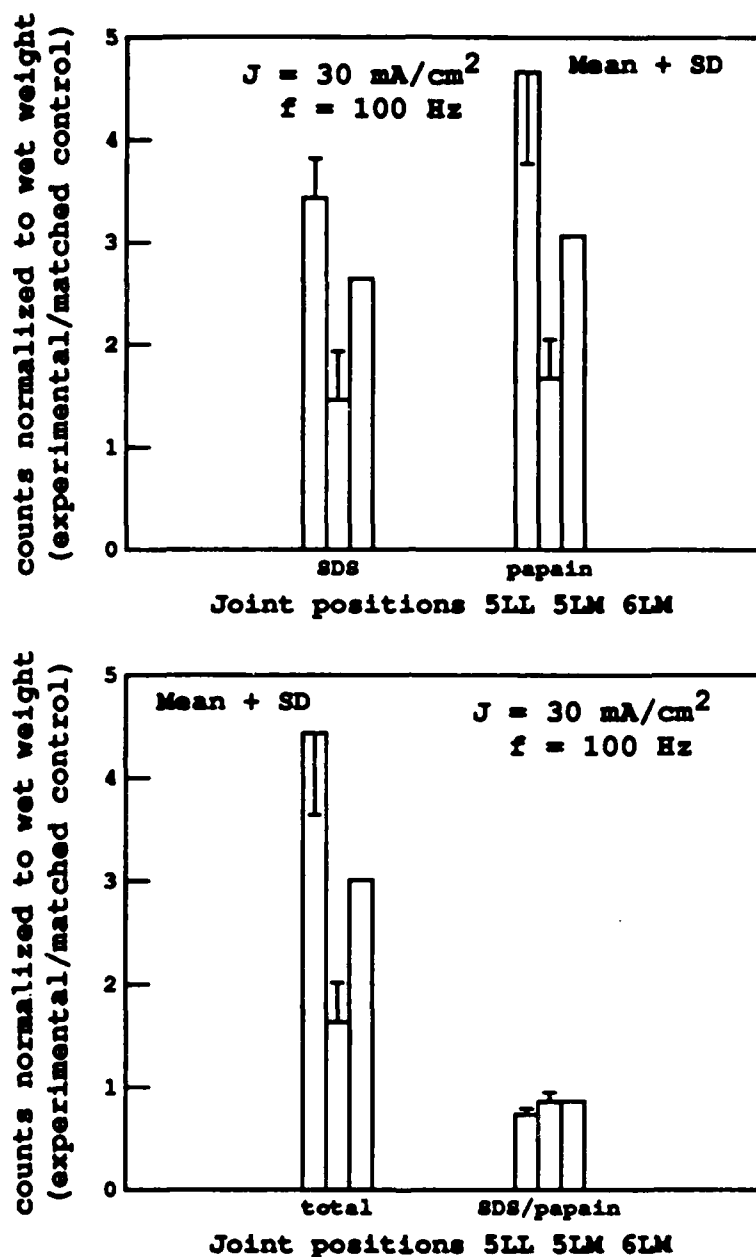


Figure 3.15: Electrical response by core position ( $f = 100 \text{ Hz}$ ,  $J = 30 \text{ mA/cm}^2$ ). Experimental/control ratios (cpm normalized to wet weight) are plotted for averages of 2 pairs of matched plugs from the same core. Where no SD bar is shown, the ratio represents data from only 1 pair.

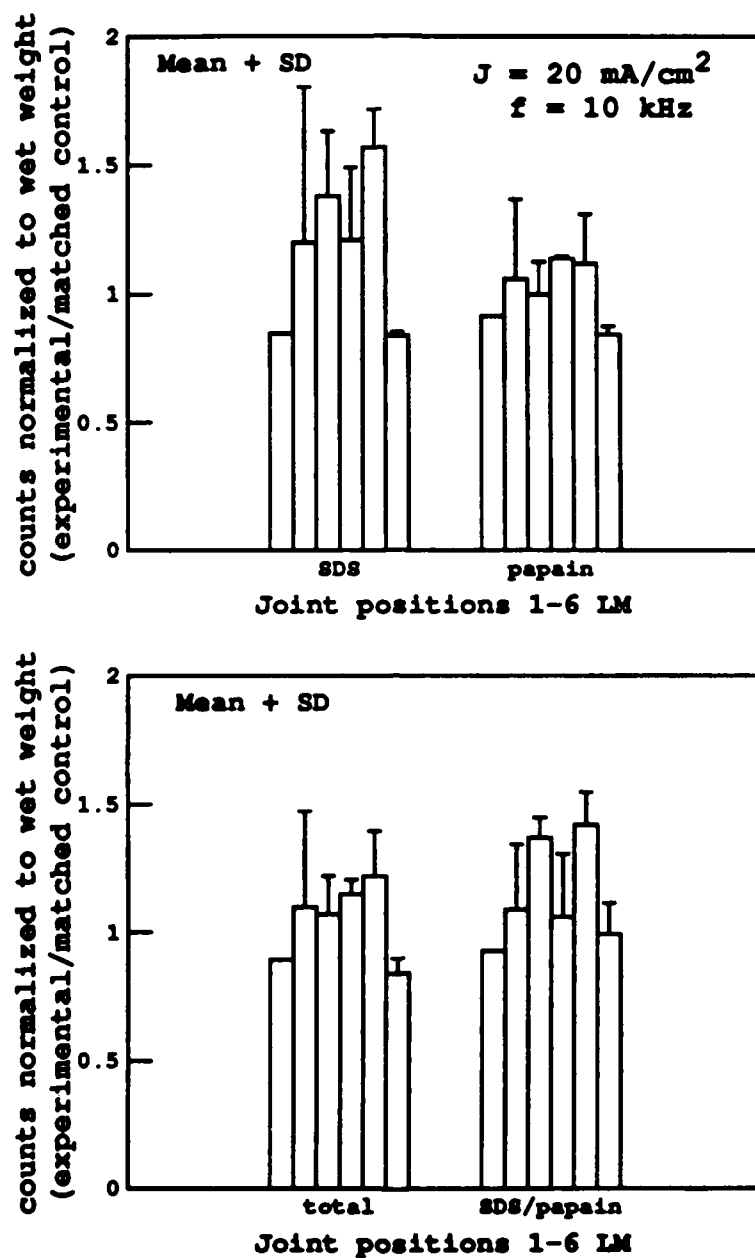


Figure 3.16: Electrical response by core position ( $f = 10 \text{ kHz}$ ,  $J = 20 \text{ mA/cm}^2$ ). Experimental/control ratios (cpm normalized to wet weight) are plotted for averages of 2 pairs of matched plugs from the same core. Where no SD bar is shown, the ratio represents data from only 1 pair.

20 mA/cm<sup>2</sup> at 10 kHz in which all plug positions from a single facet were used. (Additional plots of response by core position for other electrical experiments are included in Appendix C.4) On the lateral facet, the pattern of response with position on the joint appeared to vary more between experiments. However, the response of two pairs of plugs from a single core position on the lateral facet was also similar within a single experiment (Fig. C.4). On average, the response of electrically stimulated plugs from the lateral facet was lower than that of plugs from the medial facet. The response to electrical stimulation as a function of the joint position is summarized in Fig. 3.17 and Fig. 3.18, for all electrical experiments. Experimental to control ratios for each experiment were normalized to the mean experimental to control ratio for each experiment, and then averaged over all experiments according to position on the joint surface. Experimental to control ratios for plugs obtained from the same position on the right and left joints were not significantly different. Data for plugs from right and left joints were therefore pooled to calculate averages for each joint position. Qualitatively, protein synthesis appeared to be stimulated more in plugs from the center than from the end positions on the medial facet, although the differences were not statistically significant.

Incorporation of <sup>35</sup>S-methionine into the papain digest was stimulated more than radiolabel incorporation into SDS-soluble proteins at 100 Hz and 1 kHz. At 10 kHz, there was a larger increase in incorporation into the SDS-soluble fraction with applied field (Fig. 3.6 and 3.7). At all frequencies, experimental to control ratios for radioactivity in the papain digest and in the SDS-soluble extract seem to vary with joint position in the same way (Figs. 3.14, 3.15 and 3.16).

#### **Field Induced Increases in Incorporation were not Significantly Different for Plugs Incubated in 10%FBS versus 0.1%NS**

Control plugs incubated and labelled in 10% FBS had greater incorporation, than plugs incubated and labelled in 0.1%NS for both sets of experiments comparing

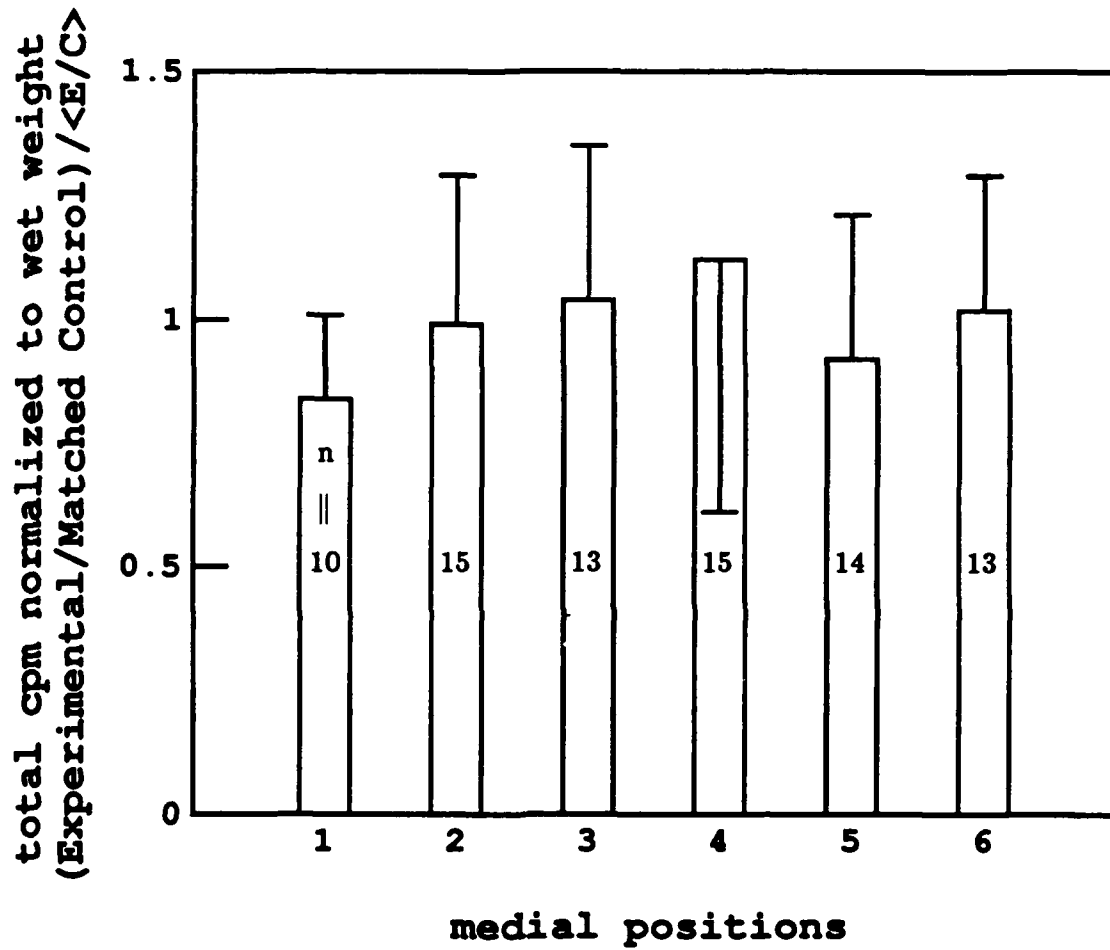


Figure 3.17: Electrical response as a function of medial joint position, averaged over all experiments.

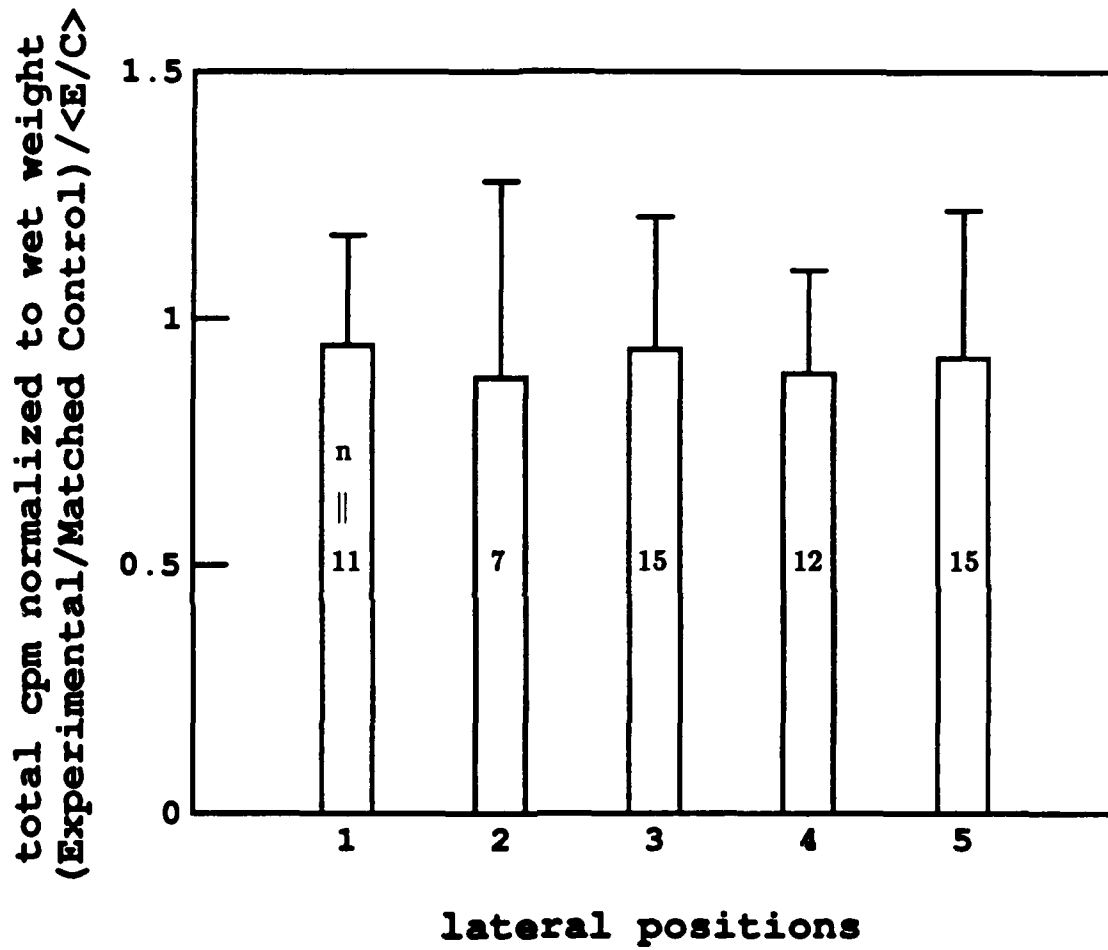


Figure 3.18: Electrical response as a function of lateral joint position, averaged over all experiments.

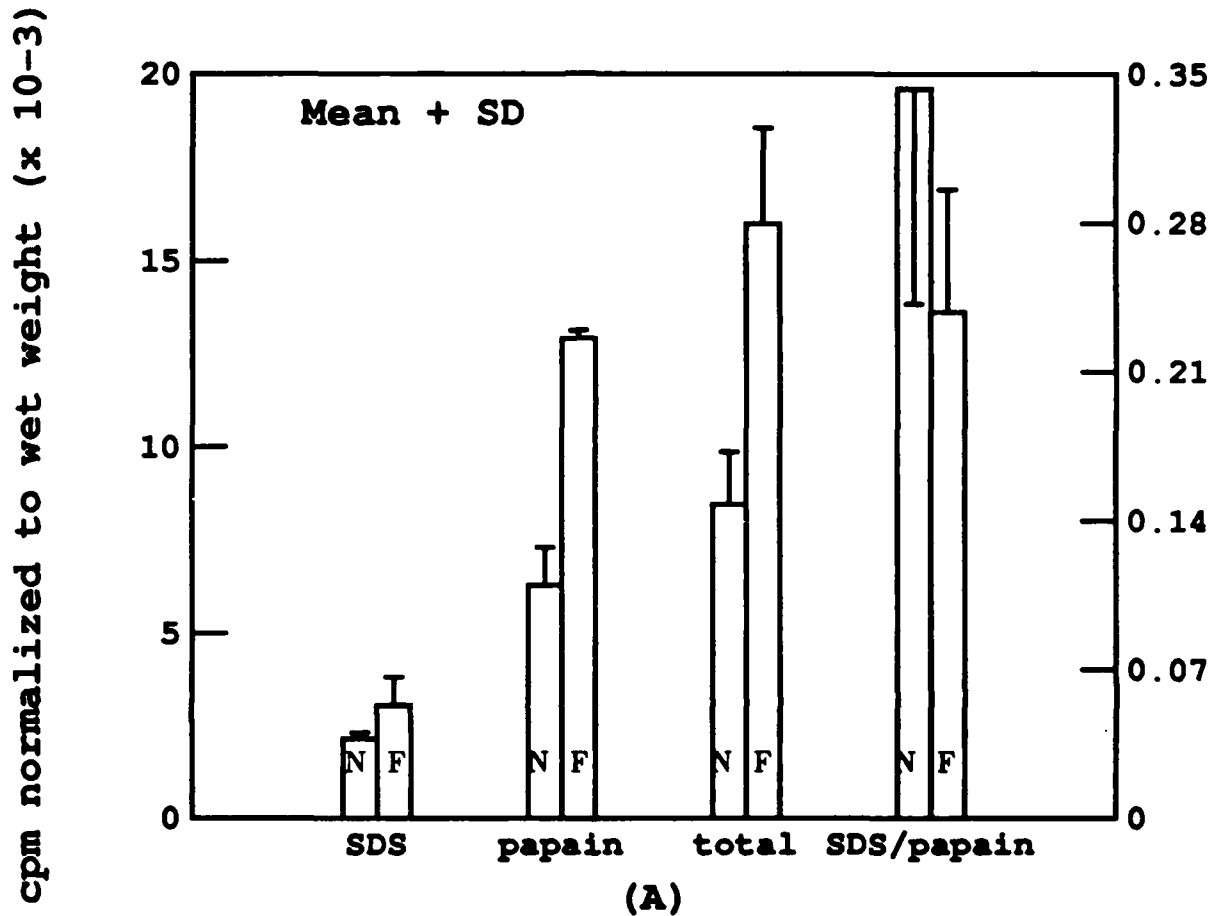


Figure 3.19: Total incorporation of  $^{35}\text{S}$ -methionine normalized to wet weight in each extract for plugs cultured and then labelled in 10%FBS, and for plugs cultured and then labelled in 0.1%NS.

(A) Incorporation in the control plugs used in electrical stimulation experiments of Fig. 3.21. Plugs from positions 2 4 and 6 on the right medial facet (2RM,4RM,6RM) were incubated in 10%FBS, and plugs from the same positions on the left medial facet (2LM,4LM,6LM) were incubated in 0.1%NS.



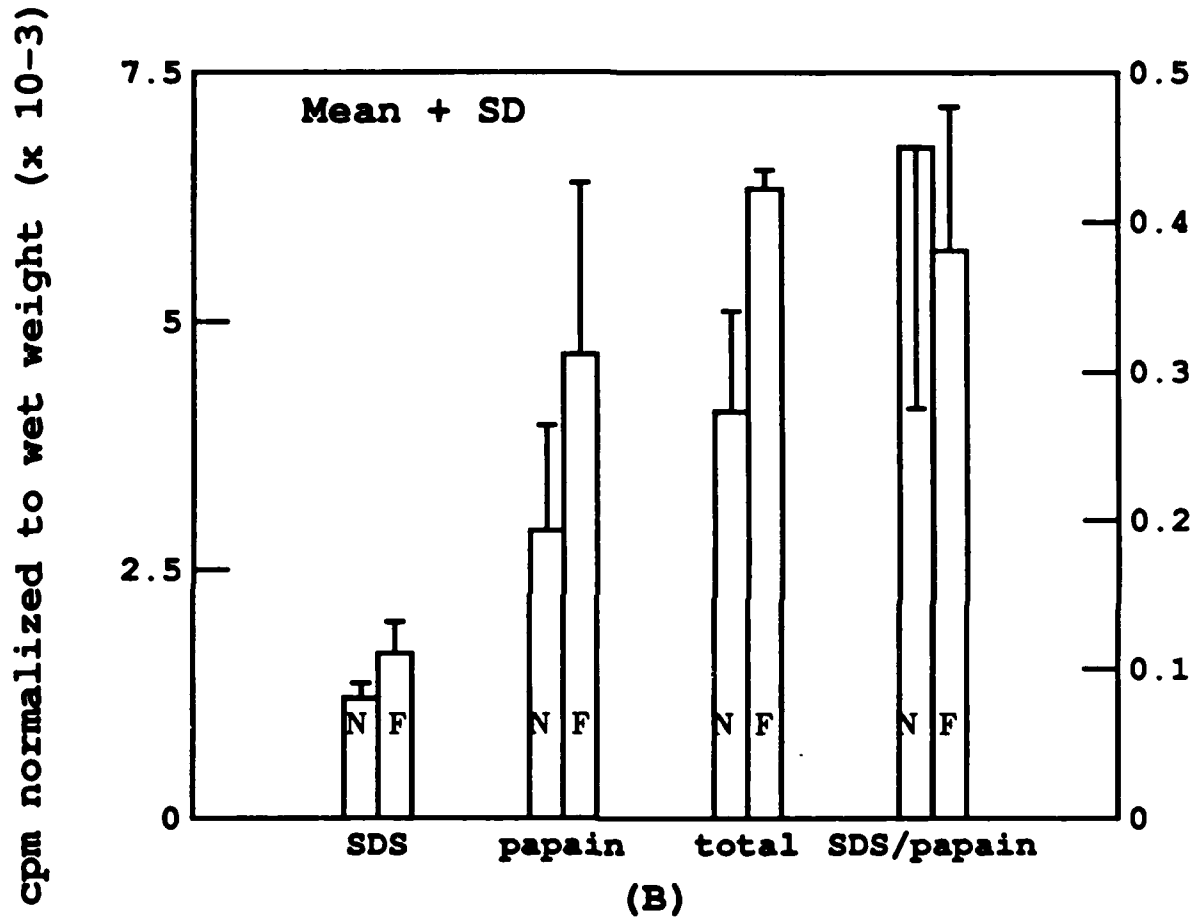


Figure 3.19: (B) Incorporation in the control plugs used in the electrical stimulation experiments of Fig. 3.22. Plugs obtained from the same core positions, but opposite joints were incubated in the two different serum concentrations.

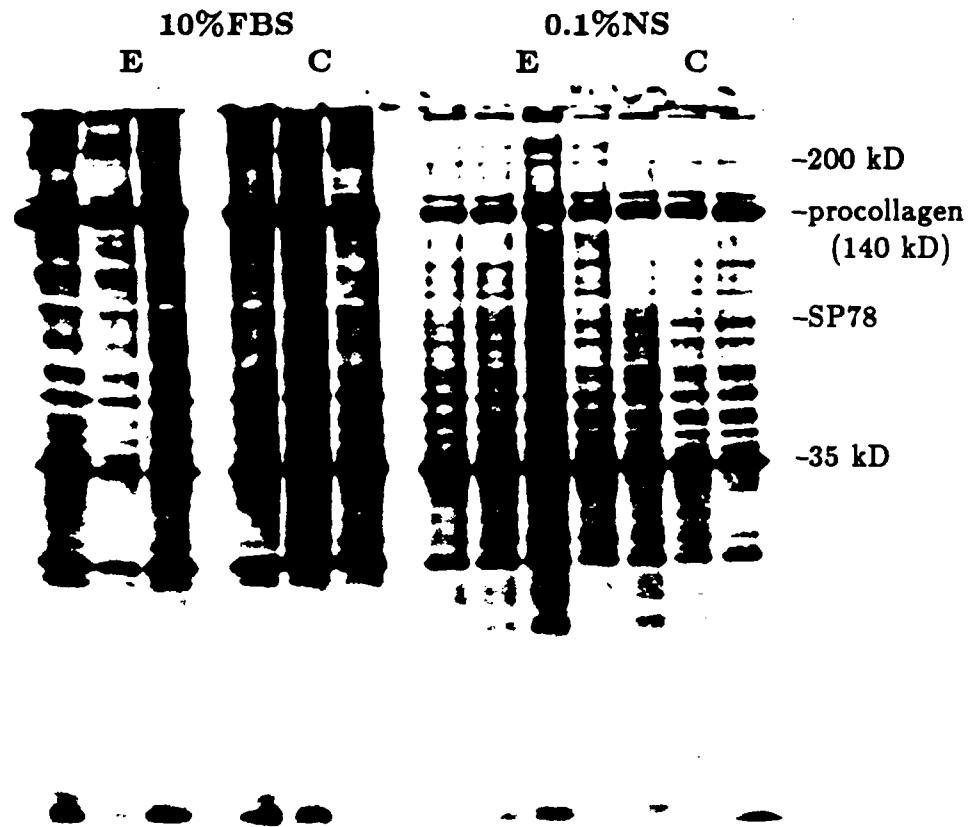


Figure 3.20: Fluorograph of cartilage plugs stimulated and labelled for 12 hours at 20 mA/cm<sup>2</sup> at 100 Hz. Plugs corresponding to the lanes marked 10%FBS and 0.1%NS were stimulated and labelled in DMEM-F and DMEM-N, respectively. The lanes marked E and C correspond to electrically stimulated plugs, and their matched controls respectively.

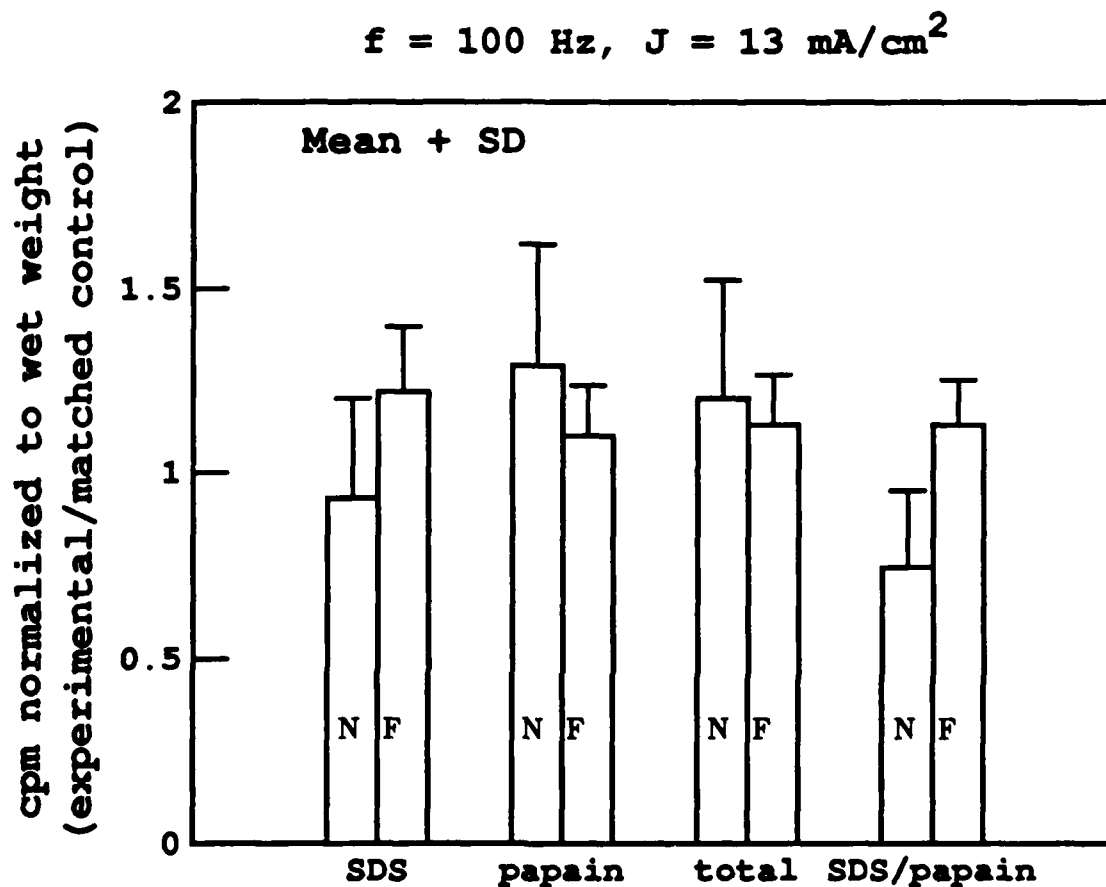


Figure 3.21: The ratio of incorporation (electrically stimulated/control plugs) is plotted for an electrical experiment ( $J = 13 \text{ mA/cm}^2$  and  $f=100 \text{ Hz}$ ), in which plugs were incubated and labelled in DMEM-F (10%FBS) or in DMEM-N (0.1%NS) during stimulation. Inside the bars N indicates plugs incubated in NS, and F indicates plugs incubated in FBS. Plugs from the right medial facet (2RM,4RM and 6RM) were incubated and labelled in 10%FBS, and plugs from the left medial facet (2LM,4LM and 6LM) were incubated in 0.1%NS.

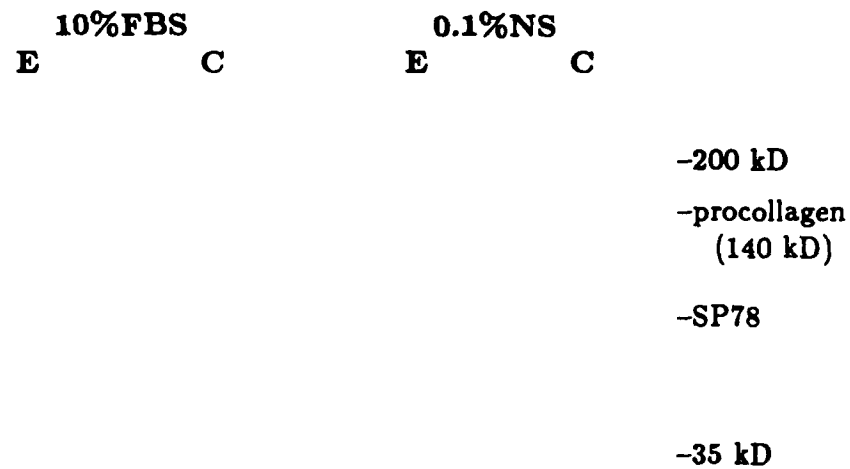


Figure 3.20: Fluorograph of cartilage plugs stimulated and labelled for 12 hours at 20 mA/cm<sup>2</sup> at 100 Hz. Plugs corresponding to the lanes marked 10%FBS and 0.1%NS were stimulated and labelled in DMEM-F and DMEM-N, respectively. The lanes marked E and C correspond to electrically stimulated plugs, and their matched controls respectively.

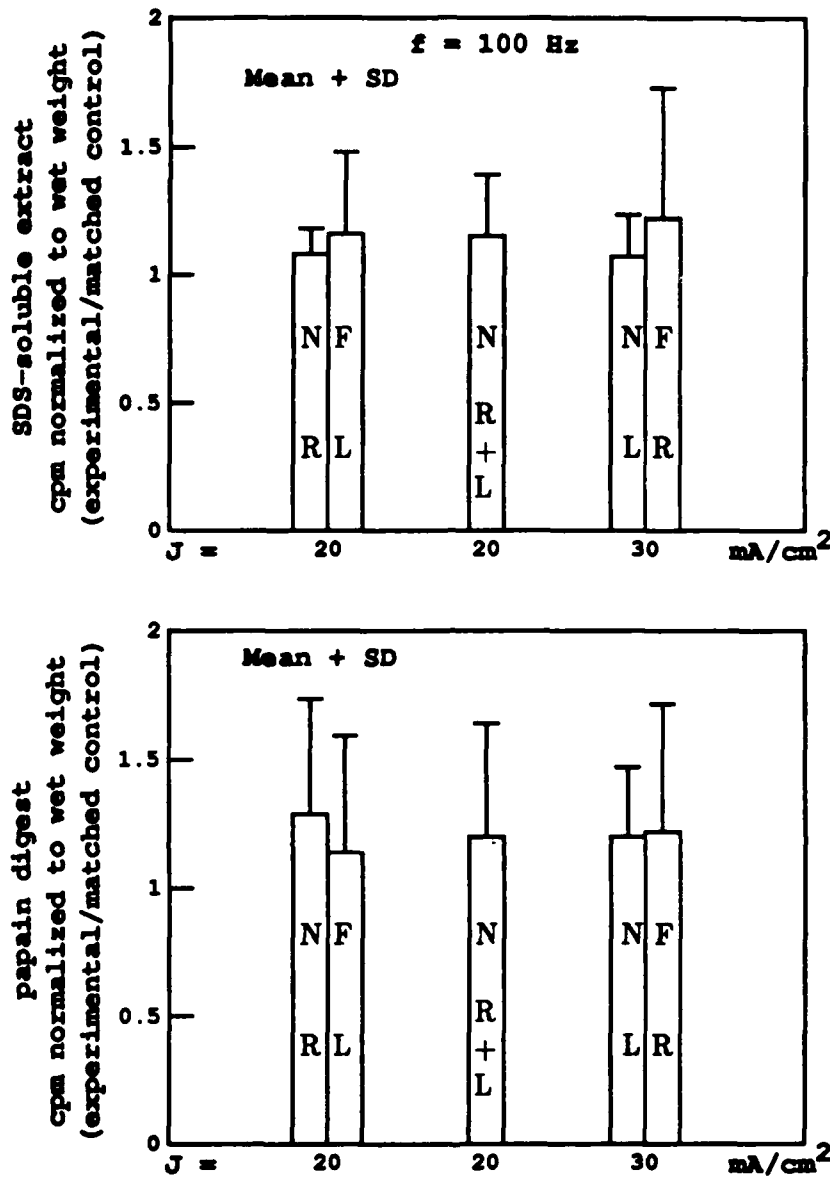


Figure 3.22: The ratio of incorporation in electrically stimulated (experimental) to control plugs ratios for plugs incubated in 10%FBS compared to plugs incubated and labelled in 0.1%NS. Inside the bar N indicates plugs incubated in NS, and F indicates plugs incubated in FBS. R and L indicate the joint side from which plugs were obtained. The paired bars represent experiments in which plugs from the same joint position were used for both plugs incubated in 10%FBS and in 0.1%NS, but from R or L joints as indicated. The single bar includes plugs from both right and left joints. It represents an experiment done in 0.1%NS to test that core positions on right and left joints were equivalent in terms of response to electrical stimulation.

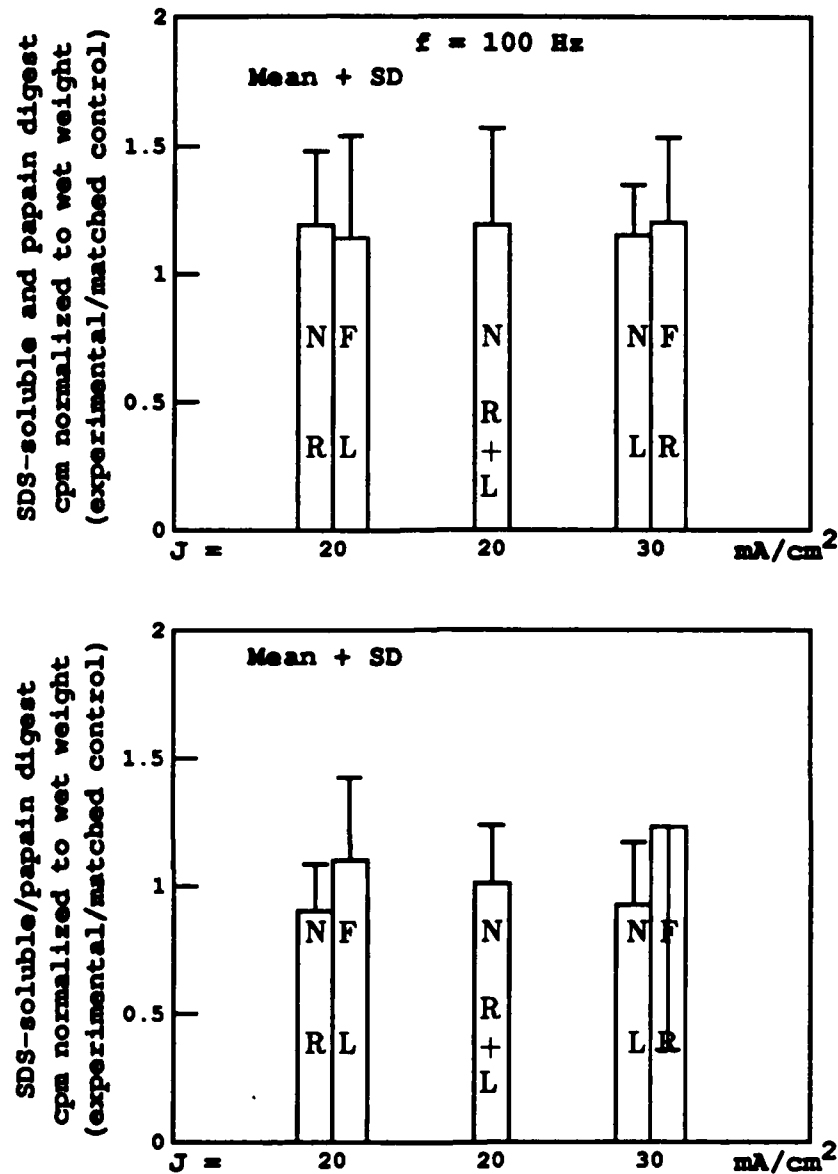


Figure 3.22: The ratio of incorporation in electrically stimulated (experimental) to control plugs ratios for plugs incubated in 10%FBS compared to plugs incubated and labelled in 0.1%NS. Inside the bar N indicates plugs incubated in NS, and F indicates plugs incubated in FBS. R and L indicate the joint side from which plugs were obtained. The paired bars represent experiments in which plugs from the same joint position were used for both plugs incubated in 10%FBS and in 0.1%NS, but from R or L joints as indicated. The single bar represents an experiment done in 0.1%NS to test that core positions on right and left joints were equivalent in terms of response to electrical stimulation.

the effects of electrical stimulation with the two serum concentrations (Fig. 3.19). However, the increase in incorporation due to electrical stimulation, was not significantly different in plugs cultured and labelled in 10% FBS compared to plugs cultured and labelled in 0.1%NS (Figs. 3.21 and 3.22). The gel fluorographs showed no changes in protein bands in lanes corresponding to electrically stimulated plugs when compared to controls in either 10%FBS or 0.1%NS (Fig. 3.20). Protein bands were different in lanes corresponding to plugs incubated in 10%FBS when compared to 0.1%NS, as had been noted previously for culture periods of greater than 5 days (Chapter II). Fig. 3.23 shows the response in 10%FBS and 0.1%NS by core position for the second set of experiments. In 0.1%NS, the response for right and left plug positions 4LL and 4RL, and 4LM and 4RM were similar. No conclusion could be drawn regarding differences in the response by plug position in the different serum concentrations from the data available.

$f = 100 \text{ Hz}$

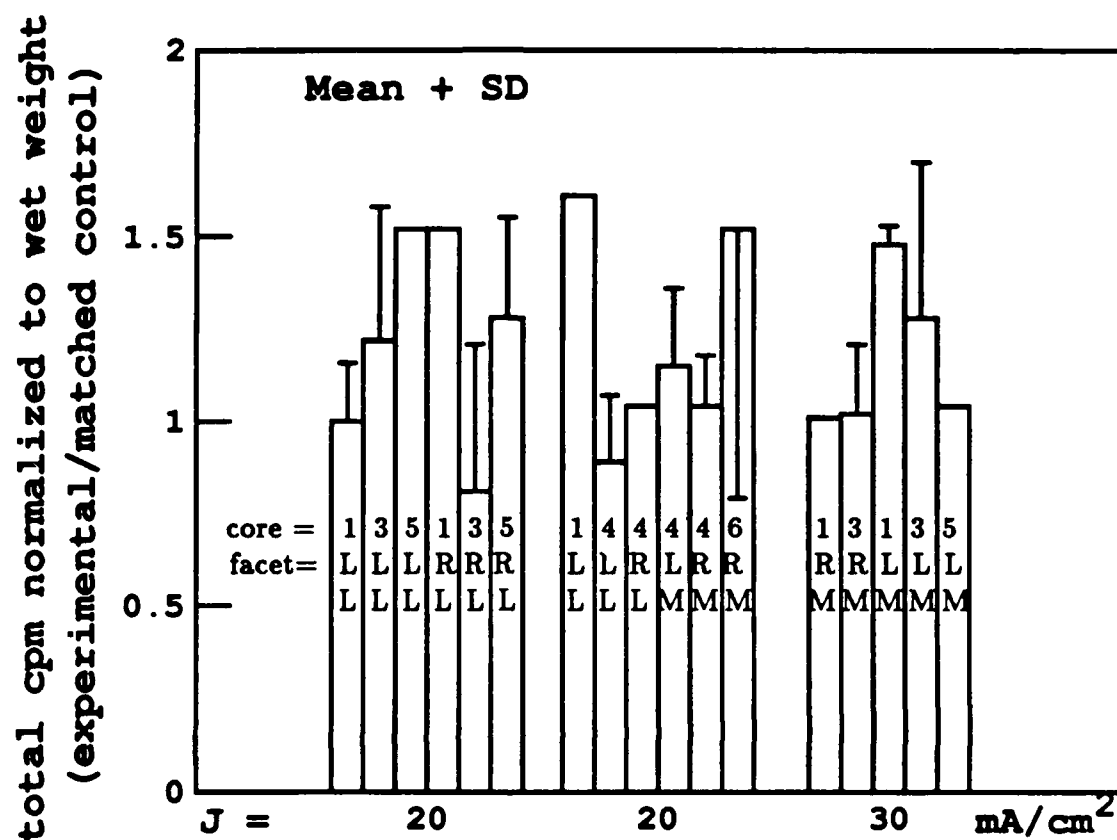


Figure 3.23: Electrical response to stimulation at 100 Hz and either 20 or 30 mA/cm<sup>2</sup> by core pairs incubated and labelled in 10%FBS and 0.1%NS. These cores are the same as those used to calculate the mean electrical response shown in Fig. 3.22.



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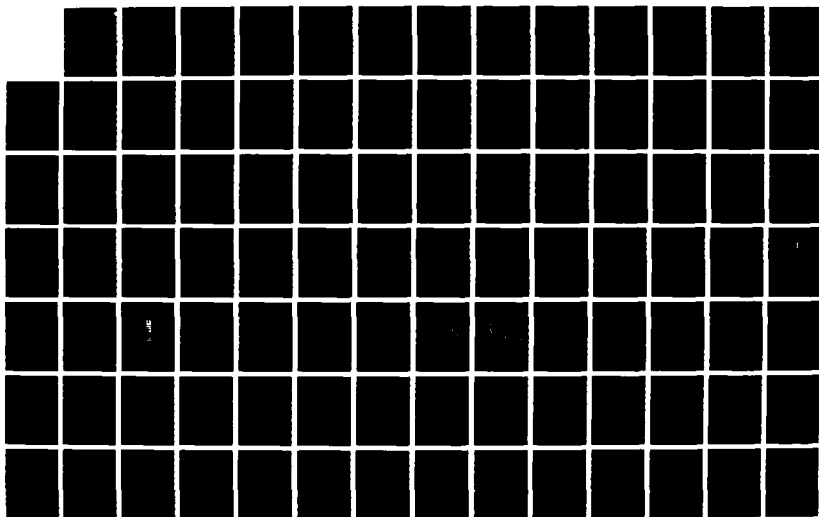
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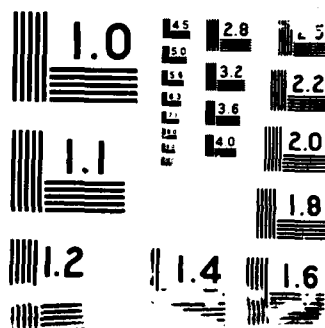
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## Chapter IV

### Experimental Model: Discussion and Conclusion

#### 4.1 Introduction

The motivation for investigating the effects of electric fields on biological tissue, using cartilage as the model tissue, was twofold. The motivation was first, to begin to elucidate the role played by physiologically generated fields in physiological function, such as in cellular metabolism. Second, it was to begin to understand how externally applied fields might alter normal physiological function, either so that controlled changes in function might be elicited by field application (as in the clinical setting) or to determine the biological hazards which might be associated with fields in the environment. The motivations are related, since the mechanisms by which physiologically generated or externally induced fields might act on tissues may be similar within the range of physiological frequencies (less than 10 kHz).

The experimental model, described in chapters II and III, was the effect of electric fields on stress protein and total protein synthesis in cartilage tissue. Electric fields are generated within articular cartilage tissue as part of normal physiological loading, so that this is an appropriate tissue in view of the first motivation—understanding the role of normal physiologically generated fields. Since chondrocytes maintain the extracellular matrix of cartilage, field-induced changes in protein synthesis could ultimately lead to a major change in physiological function.

Chapter II described control studies that were required to develop the experimental model. First, the control studies showed that induction of stress proteins by incubation at elevated temperatures was dose dependent (i.e., depended on the duration of incubation and the temperature). Second, based on these control studies, the culture conditions prior to electrical stimulation of cartilage specimens were chosen to be a minimum of 5 days culture in medium containing 0.1% NS. Under

these conditions, total protein synthesis was at a steady state for several days, during which the electrical stimulation experiments were done. The stress response to heat was also at steady state as assessed by the intensity of SP70 and SP78 on gel fluorographs and total protein synthesis during the high temperature incubation. In addition, it was shown that the stress response was not induced for incubation at temperatures below 39°C. Therefore, field induced ohmic heating would not induce stress proteins below this temperature.

Chapter III described in detail the results of experiments in which cartilage plugs were stimulated by electric fields for 12 hours at current densities up to 30 mA/cm<sup>2</sup>. The salient features of the response were:

- (1) No stress protein synthesis was induced for any field amplitude between 10 mA/cm<sup>2</sup> to 30 mA/cm<sup>2</sup> for frequencies of 1, 10, and 100 Hz and 1 and 10 kHz.
- (2) An increase in total protein synthesis, as measured by <sup>35</sup>S-methionine incorporation, was induced for high enough current densities (>10 mA/cm<sup>2</sup>).

Incorporation appeared to increase with current density at any given frequency. The increase in incorporation was greater in the papain digest than in the SDS-soluble extract at all frequencies except 10 kHz. The increase in both SDS-soluble extract and papain digest varied in nearly the same way with the position on the joint surface from which the cartilage plugs were obtained.

In this chapter, these results will be used to address the twofold motivation of understanding the effects of physiologically generated and externally applied fields. In summary, the conclusions based on the experimental results were:

- (1) The lack of stress response indicates that fields within these amplitudes and frequencies do not act to produce an accumulation of abnormal proteins that are toxic to the chondrocytes.
- (2) The increase in protein synthesis does indicate, however, that fields act on chondrocytes by another, non-toxic mechanism. The finding that current densities that are large relative to physiological current densities, are required to measurably

increase synthesis, does not necessarily indicate that physiological current densities are unimportant. This model system was too noisy to detect small (e.g.  $< 20\%$ ) changes in synthesis. Such changes can be important, particularly if the change occurs in a few specific proteins. One of the factors responsible for the large variation in electrical response was the inhomogeneity between cartilage specimens, as discussed in Chapter III.

The results, therefore, suggest that experimental modifications may be required for further understanding this field induced increase in synthesis. Modifications include: use of more homogeneous samples, with variables (e.g. cell density, matrix composition) that can be controlled, such as chondrocytes cultured in agar gels; and chronic stimulation by lower current densities (longer than 12 hours).

#### **4.2 Applied Current Densities Do Not Induce Stress Response**

No synthesis of any of the stress proteins identified by incubating cartilage plugs at elevated temperatures was induced by electrical stimulation. No change was observed in any gel fluorograph between the intensity of any bands in lanes corresponding to cartilage samples in the electrically stimulated chamber, relative to the intensity of bands in lanes corresponding to cartilage samples in the control chamber. This lack of stress response is important since it indicates that current densities up to  $10 - 30 \text{ mA/cm}^2$  in the frequency range  $1 \text{ Hz} - 10 \text{ kHz}$  do not produce a toxic accumulation of abnormal proteins by any mechanism.

SP70 was induced in all cartilage plugs incubated at elevated temperatures ( $>41^\circ\text{C}$ ) that were obtained from the same cows used in electrical experiments. The lack of stress response, therefore, cannot be attributed to an inability of particular specimens to produce stress proteins.

Thus, as regards concern with possible hazardous effects of electric fields in the environment, the lack of stress response indicates that local current densities in the region of the cells within this frequency and amplitude regime are not toxic

to cartilage tissue, as defined by the stress response. Since the stress response is induced by a variety of toxic agents and is not mechanism specific, stress response is a reasonably general definition for toxicity. Furthermore, the maximum current density applied ( $30 \text{ mA/cm}^2$ ) is at least an order of magnitude larger than fields applied clinically, and is many orders of magnitude larger than fields expected to be induced by environmental sources for frequencies less than 10 kHz.

In addition, the lack of stress response is important in the electrical experiments, since stress protein synthesis serves as a control for ohmic heating (above  $39^\circ\text{C}$ ) and for electrode reaction products. The lack of stress response, therefore, indicates that the increase in protein synthesis in cartilage plugs exposed to electric fields is not due to significant ohmic heating, and is not due to electrode reaction products.

#### **4.2.1 Sensitivity of the SDS-PAGE Assay**

Very low levels of stress protein induction would not be observed by the method of SDS-PAGE, followed by fluorography and examination of the gel fluorograph. Stress protein synthesis was not observed for temperatures below  $39^\circ\text{C}$ . However, the SP70 band corresponding to plugs preincubated at  $39^\circ\text{C}$  prior to incubation at  $43^\circ\text{C}$  was less intense than the band corresponding to plugs incubated at  $37^\circ\text{C}$  prior to incubation at  $43^\circ\text{C}$  (chapter II). The lower level of SP70 with preincubation at  $39^\circ\text{C}$  suggests that low levels of SP70 may be produced at this temperature, which are not visualized on the fluorographs.

#### **4.2.2 The Stress Response as a Control**

In control studies, stress protein synthesis was induced for temperatures above  $41^\circ\text{C}$  and not at temperatures below  $39^\circ\text{C}$ . Madreperla et al. [131] and Kubo et al. [111] found that stress proteins were induced, as visualized on gel autoradiographs for temperatures above  $39^\circ\text{C}$ . Thus, the lack of stress response in the electrical ex-

periments demonstrates that temperatures did not rise above 39°C. Temperature measurements showed that, in fact, the increase in temperature due to ohmic heating at the largest current density (30 mA/cm<sup>2</sup>) was less than 0.2°C.

SP70 served also as a control for electrode reaction products since it had been shown to be induced by electrode reaction products (Chapter III). SP70 was induced in plugs incubated in conditioned media obtained from the chamber adjacent to the electrodes following an electrical experiment. Total protein synthesis was also lower in these plugs. In pilot studies (described in Appendix C.3), SP70 was induced in plugs located adjacent to the electrodes in 6 of 11 experiments not using agar bridges to separate the cartilage plugs from the electrodes. Stimulation was for 6 hours in the majority of these experiments, and current densities ranged from 1 to 4 mA/cm<sup>2</sup> for 1/4 inch plugs (equivalent to 5 - 20 mA/cm<sup>2</sup> for 3 mm plugs) at frequencies of 1 Hz, 10 Hz and 1 kHz. The frequent induction of SP70 in these experiments using short stimulation times and low current densities suggests that SP70 induction is a sensitive indicator of electrode reaction products.

Following all electrical experiments described in chapter III, media was removed from the chamber near the ends and used to incubate fresh plugs. SP70 was not induced in plugs incubated in media from the chamber following electrical experiments, with one exception. (For the one exception, end plugs were excluded in calculating the mean response). Total protein synthesis in plugs incubated in the conditioned media was not different from controls incubated in fresh media in any experiment. Thus stress protein synthesis and total protein synthesis both indicate that the agar bridges effectively isolated the cartilage plugs from the electrodes, and that the observed increase in total protein synthesis was not due to electrode artifacts.

#### **4.2.3 No Field Induced Changes in Other Specific Proteins**

Changes in synthesis of specific proteins other than stress proteins might also be induced by applied current densities. No change in any bands was observed by comparing lanes on the gel fluorographs corresponding to stimulated and control plugs. However, as in the case of stress proteins, it is possible that small changes in the synthetic rate in specific proteins or that low levels of new proteins might be induced, which cannot be observed by 1 dimensional gel electrophoresis. One method to measure very small changes or low levels of new protein synthesis is 2-D gel electrophoresis [70]. In 2-D electrophoresis, proteins are separated by their native isoelectric point as well as by molecular weight.

### **4.3 Field Induced Increase in Total Protein Synthesis**

#### **4.3.1 Introduction**

Total protein synthesis measured by total incorporation of  $^{35}\text{S}$ -methionine, increased in cartilage plugs stimulated by high enough current densities relative to their matched controls. Electric fields, therefore, appear to act by some mechanism to change an important physiological function of the chondrocytes. The increase in incorporation depended on the position on the joint surface from which plugs were obtained, which resulted in a large variation in the response to electric fields within a single experiment. The mean increase in incorporation, however, increased with the magnitude of the current density. The proportion of incorporation in the SDS-soluble extract relative to the papain digest extracellular decreased with the magnitude of the current density, except at 10 kHz.  $^{35}\text{S}$ -methionine incorporation into the SDS-soluble extract represented incorporation into intracellular proteins and some extracellular proteins that were not covalently bound to the matrix and were able to diffuse out of the matrix.  $^{35}\text{S}$ -methionine incorporation in the papain digest represented incorporation into extracellular proteins which were then cova-



lently bound within the extracellular matrix or proteins that were unable to diffuse out.

The increase in total synthesis, and the decrease in incorporation in the SDS-soluble extract relative to the papain digest is not due either to a significant rise in temperature due to ohmic heating, or to electrode reaction products.

The change in total protein synthesis and in the ratio of SDS-soluble to papain digested proteins indicates that electric fields may play a role in physiological regulation of chondrocyte metabolism, and may furthermore have important implications regarding clinical applications of fields to alter growth and remodeling.

#### 4.3.2 Variation with Amplitude

Total incorporation in electrically stimulated plugs relative to control plugs (Fig. 3.8) increased monotonically with current density within a given frequency, with two exceptions ( $f = 1$  Hz, between 10-20 mA/cm<sup>2</sup>, and  $f=100$  Hz, between 13-20 mA/cm<sup>2</sup>). The ratio of incorporation in the SDS-soluble extract relative to the papain digest similarly decreased with current density, except at 10 kHz. At the two mid frequencies, (100 Hz and 1 kHz) the decrease in the ratio resulted from greater field induced stimulation of radiolabel incorporation of <sup>35</sup>S-methionine into the papain digest than the SDS-soluble extract as seen by plotting the two fractions with their sum and ratio, within a single frequency (e.g. Fig. 4.1). At the low frequencies (1 and 10 Hz), the field induced changes in the two extracts with current density which result in a decrease in the ratio with current density is not as straightforward. At these two frequencies, while the ratio decreased with current density, the decrease was from mean values for the ratio that were greater than 1 (1 is equivalent to equal ratios for control and experimental plugs) down to a ratio of approximately 1 at 30 mA/cm<sup>2</sup>, rather than decreasing from approximately 1 to lower values, as at 100 Hz and 1 kHz. The decrease in the ratio at 1 Hz, was made up of a greater increase in SDS-soluble incorporation at low current densities,

$f = 100 \text{ Hz}$

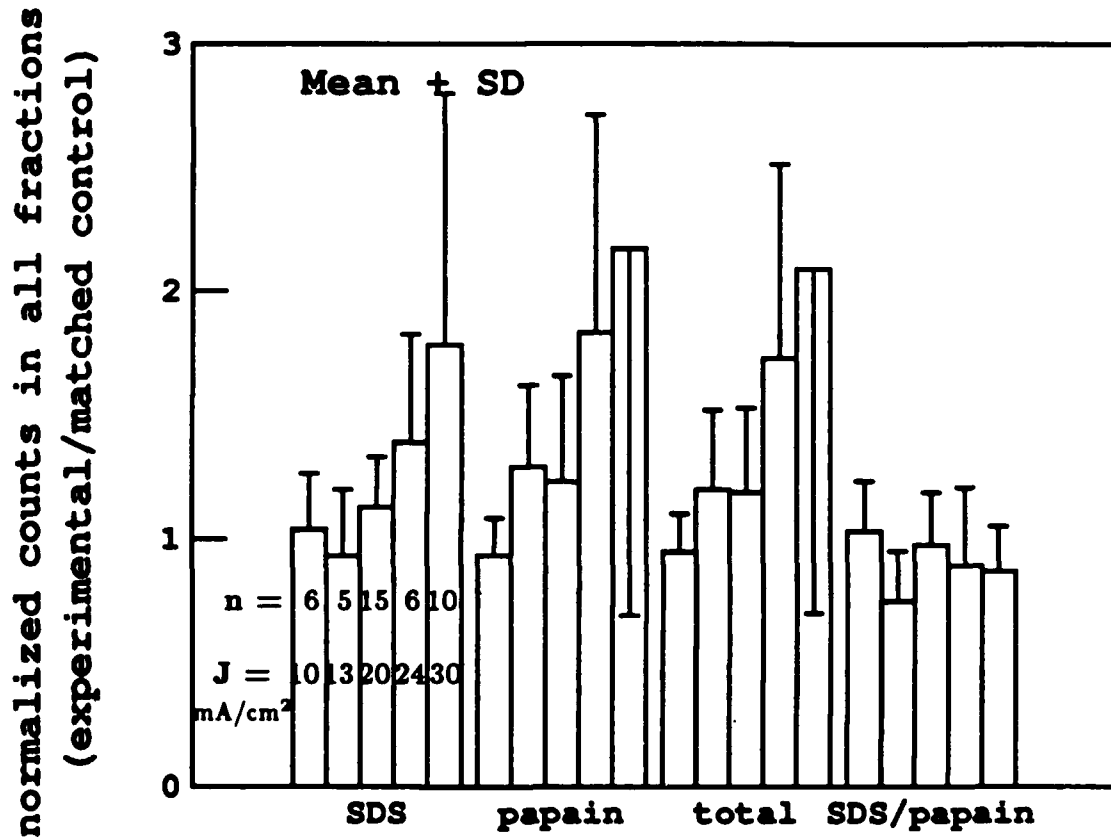


Figure 4.1: Electrical response in SDS and papain extracts as a function of amplitude at  $f = 100 \text{ Hz}$ . The mean counts in each extract normalized to wet weight, are plotted as the ratio for experimental plugs to their matched controls.

$f = 10 \text{ kHz}$

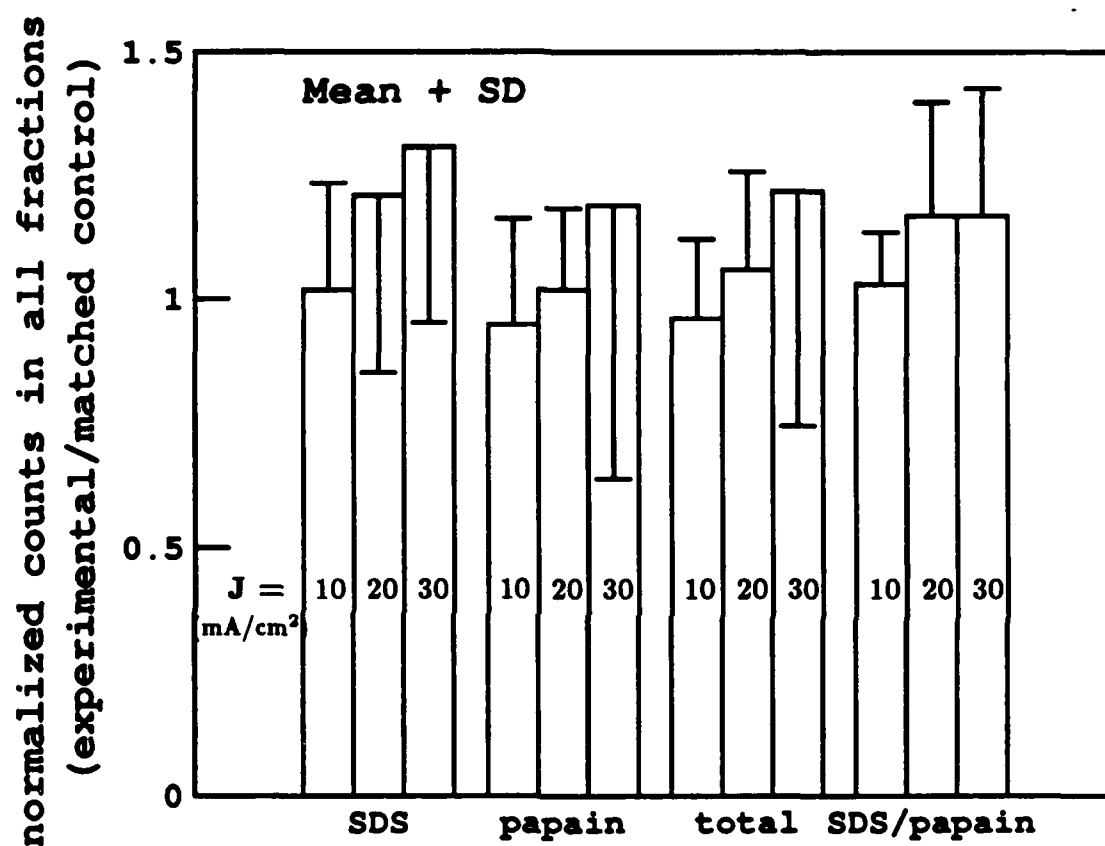


Figure 4.2: Electrical response in SDS and papain extracts as a function of amplitude at  $f = 10 \text{ kHz}$ . The mean counts in each extract normalized to wet weight, are plotted as the ratio for experimental plugs to their matched controls.

and greater incorporation in the papain digest at high current densities. Thus, although the ratio decreased for stimulation at these two frequencies as for 100 Hz and 1 kHz, the field induced changes in the two fractions resulting in the decrease in ratio, may be different. At 10 kHz, there was a larger increase in incorporation into the SDS-soluble fraction with applied field as can be seen in Fig. 4.2.

At 100 Hz and 1 kHz, the increase in total incorporation synthesis and the decrease in the ratio of SDS-soluble to papain digest incorporation are consistent both with an increased level of synthesis, and with an increase in covalent binding of the extracellular matrix proteins. An increase in synthesis of all proteins, including both proteins which are normally retained within the cell and those secreted into the extracellular space would be expected to result in a concomitant increase in export of the extracellular matrix proteins. Accumulation of intracellular proteins would presumably be regulated, whereas accumulation of protein within the extracellular matrix would not (at least within the short exposure times investigated in this study). Even in the absence of an increase in binding rate, therefore, labelled proteins bound to the extracellular matrix could accumulate proportionally more than the labelled intracellular proteins. An increase in extracellular binding rate would further enhance this difference. Such an increase might result from an increase in the extracellular concentration of enzymes that catalyze covalent binding of proteins to the matrix. It is worth noting that enhanced total protein synthesis in cartilage plugs cultured in 10%FBS relative to plugs cultured in 0.1%NS is accompanied by a lower SDS-soluble to papain digest ratio (Fig. 3.19). These results may support the hypothesis that an increase in total protein synthesis results in a decrease in the ratio of incorporation in the SDS extract relative to the papain digest. However, in the case of culture in 10%FBS vs 0.1%NS, there were also differences in synthesis of specific proteins. In particular, cartilage plugs incubated in 10%FBS had higher levels of procollagen synthesis.

#### 4.3.3 Variation with Joint Position and with Cow

The largest increases in total protein synthesis at the highest current densities are, in general, associated with the largest standard deviations. These larger standard deviations appear to be due to the field accentuating differences between plugs from different positions on the joint surface. Fig. 3.14 and 3.15, for example, showed the electrical response by core position for two of the experiments with the largest increase and the largest standard deviations.

The standard deviations in the ratio of SDS-soluble to papain digest incorporation, in contrast, qualitatively do not appear to depend on the magnitude of the decrease, and are in general smaller than the standard deviations for experimental-to-control ratios of total protein synthesis. This observation seems reasonable, since in the ratio of SDS-soluble to papain digest incorporation, each plug has an internal control. Thus, the ratio of incorporation in the SDS-soluble extract relative to the papain digest may be a valuable parameter in assessing response to applied fields. The response of the ratio to electric field application, however, also depends on plug position (see, for example, Fig. 3.14, 3.15 and 3.16).

Differences by plug position are important because:

- (1) The large differences in response between plugs may obscure a response to lower current densities, and
- (2) The pattern of response with joint position may yield information relevant to the mechanism of interaction by identifying some factors, such as base levels of biosynthesis and matrix composition, which might influence the response.

Little increase in synthesis was found in response to current densities of 10 mA/cm<sup>2</sup>, except at 1 kHz. However, within a single experiment (e.g., Fig. 4.3), it appears that incorporation in plugs from some positions does increase in response to field stimulation, although the mean incorporation is not much different from control values.

The variation in response with position on the joint surface might be asso-

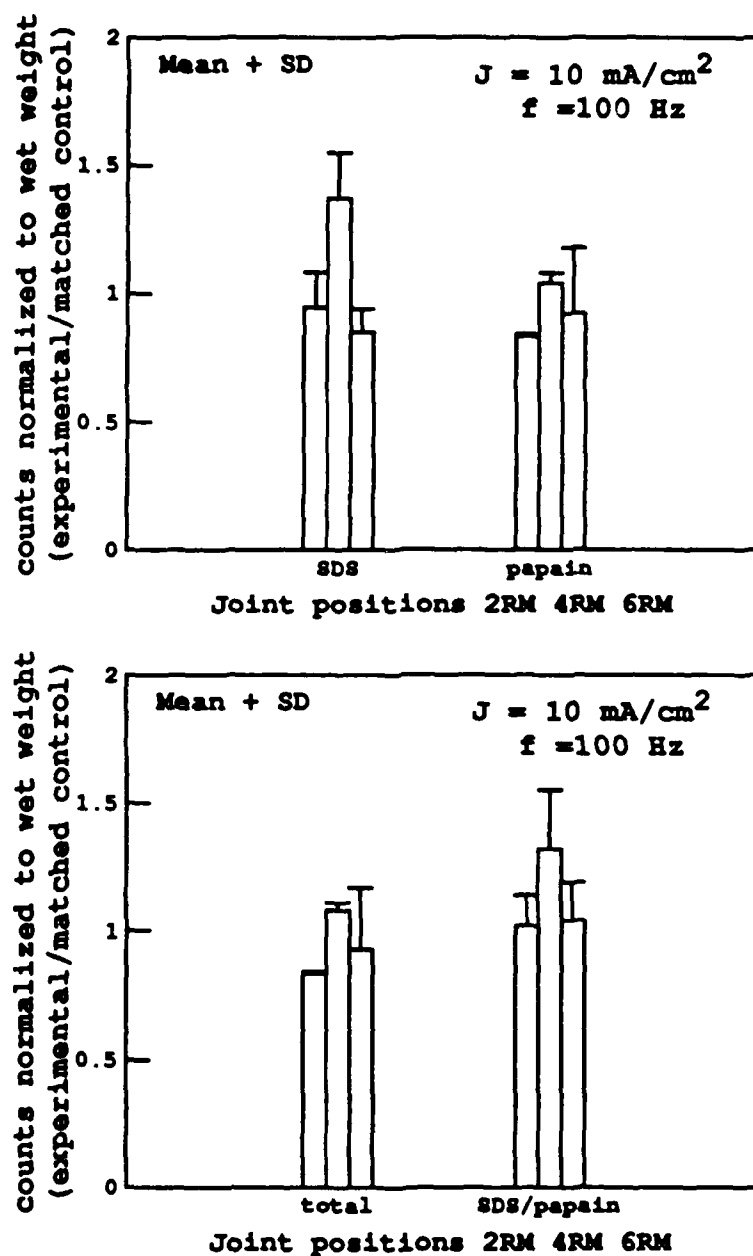


Figure 4.3: Electrical response by core position ( $f = 100$  Hz,  $J = 10$  mA/cm<sup>2</sup>). Experimental/control ratios (cpm normalized to wet weight) are plotted for averages of 2 pairs of matched plugs ( $n=2$ ).

ciated with a number of other factors that vary with position on the joint surface (Chapter I, Cartilage Morphology) and with mechanical loading. These include basal biosynthetic levels (in the absence of electrical stimulation), matrix composition and structure, and matrix cellularity. The increase in total incorporation in response to applied current densities, on average, was greatest for plugs obtained from the central positions of the medial joint surface, and least for plugs obtained from the end positions. The response exhibits a pattern with position that has been observed previously [177,53] in biosynthetic variation (total incorporation in non-stimulated plugs) over the joint surface. Hydration had also been observed to vary over the joint surface but with a slightly different pattern. Thus, plug positions on the medial facet which show the greatest response to electric fields also seem to exhibit the highest control levels of synthesis. These positions also correspond to the region of greatest mechanical loading in the joint in vivo.

Plugs from the lateral facet responded less to electric fields than plugs from the medial facet (Fig. 3.18). The average response of plugs from the lateral facet may not demonstrate an obvious pattern with position over the joint surface partly due to the fact that these plugs are associated with a lower level of response, so that the field is not accentuating differences between plugs. Plugs from the lateral facet had previously been found to have lower control levels of synthesis relative to plugs from the medial facet (Appendix B [177]). Thus, the facet with the smaller response to electric fields also seems to exhibit the lowest control levels of synthesis.

The pattern of electrical response with position on the medial joint surface, and the lower response on the lateral facet relative to the medial facet, both suggest that the electrical response is greatest for the highest basal level of biosynthesis (control plug synthesis). Therefore, a higher basal level of synthesis produced by an increase in serum concentration during culture might similarly enhance the response to electric fields. As shown in Chapter II, culture in 10% FBS increased control levels of synthesis relative to 0.1%NS (Fig. 3.19). However, there was no apparent

difference in electrical response using the two serum concentrations. The lack of a different response in the two serum concentrations does not eliminate the possibility that a greater response to fields is associated with a greater basal level of synthesis. First, the differences exhibited with position on the joint surface are small enough that a larger number of samples would be required to observe differences on that order (number = 40 to observe a 10% change, at  $p < 0.05$ , for typical  $sd = 30\%$ ). Second, culture in 10%FBS may not be optimum for observing differences in response to fields due to basal synthetic levels. As discussed in relation to choosing the culture conditions for the electrical experiments, 0.1%NS was chosen so that the cartilage plugs were not already near their maximum rate of synthesis. In 10%FBS, the cartilage plugs may be approaching their maximum rate of synthesis and thus be less able to increase their synthetic rate in response to fields. Culture in an intermediate serum concentration (e.g., near 1%) might best emphasize differences in response due to basal synthetic rates.

The same parameters, such as basal biosynthesis, matrix composition, and cellularity which vary with position on the joint surface, are also likely to vary with cow from which cartilage plugs are obtained. The age of the cows used varies between 1-2 weeks (saddle weights of 23 - 29 lbs), and it is probable that other important factors such as nutrition and mechanical loading of the joints prior to slaughter are not the same. Duplicate experiments were done using cartilage plugs from the same cow, and from two different cows. The increase in total incorporation in response to applied current densities was not much different for the two sets of two experiments using plugs from the same cow, but was significantly different ( $p < 0.05$ , by t test) for two experiments using plugs from two different cows (Fig. 4.4).

The decrease in the ratio of incorporation into the SDS-soluble extract/papain digest, however, was not much different for the experiments using the two different cows (no significance, Fig. 4.5). The insensitivity of this ratio to cow to cow variation in this one case, again suggests that the ratio, because of



some internal normalization may be at least as valuable a marker as total protein synthesis in assessing biosynthetic response to electric fields.

In conclusion, the variation in the response to electric fields with plug position on the joint surface, and with cow, suggests a modification of the model system used here to investigate field effects on tissues. Chondrocytes cultured in agarose, by eliminating position dependent variation, might be more sensitive to small changes induced by lower current densities. Kimura and coworkers [109] recently found that GAG synthesis in a homogeneous chondrosarcoma cell line cultured in agarose increased in response to fields as low as  $500\mu\text{A}/\text{cm}^2$ , as assessed by sulfate incorporation. Furthermore, agarose culture of chondrocytes would make it possible to vary the factors, such as cell density and matrix composition, which might influence the response to an electric field in a more controlled fashion.

#### **4.3.4 Comparison with Heat Induced Changes in Synthesis**

Temperature measurements during electrical stimulation indicated that the field induced increase in synthesis was not due to ohmic heating. Control studies showed that, in addition, the response to incubation at high temperatures was the reverse of the field induced response. At temperatures below  $39^\circ$  no change in synthesis was observed relative to controls at  $37^\circ\text{C}$  for up to 6 hours (Chapter II). For 3 hour incubations at  $41^\circ$  and  $43^\circ\text{C}$ , on day 7 after tissue excision, (a day used for electrical experiments) total synthesis decreased relative to controls (Fig. 4.6 A), and the ratio of SDS-soluble/papain digest incorporation increased (Fig. 4.6 B).

#### **4.3.5 Variation with Frequency**

The increase in total protein synthesis and the decrease in the SDS-soluble/papain ratio both exhibit a suggestive peak, or dip respectively, in the range of 100 Hz-1 kHz for current densities of 20 and  $30\text{ mA}/\text{cm}^2$  (Fig. 3.11 and 3.12). The contribution by the SDS-soluble and papain digest fractions to the decrease in their ratio may

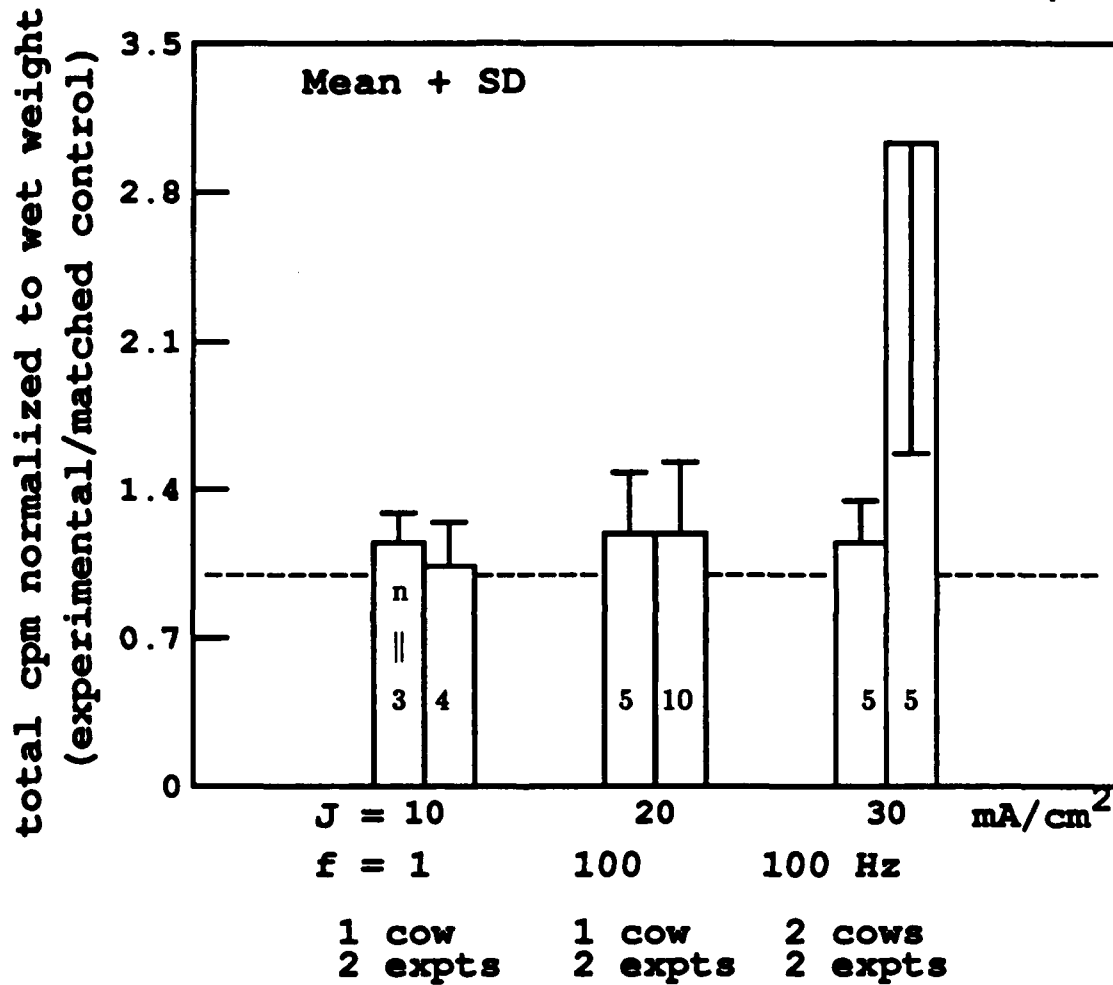


Figure 4.4: Variation in total incorporation by cow for the same electrical stimulation protocol (frequency and current density as labelled in the figure).

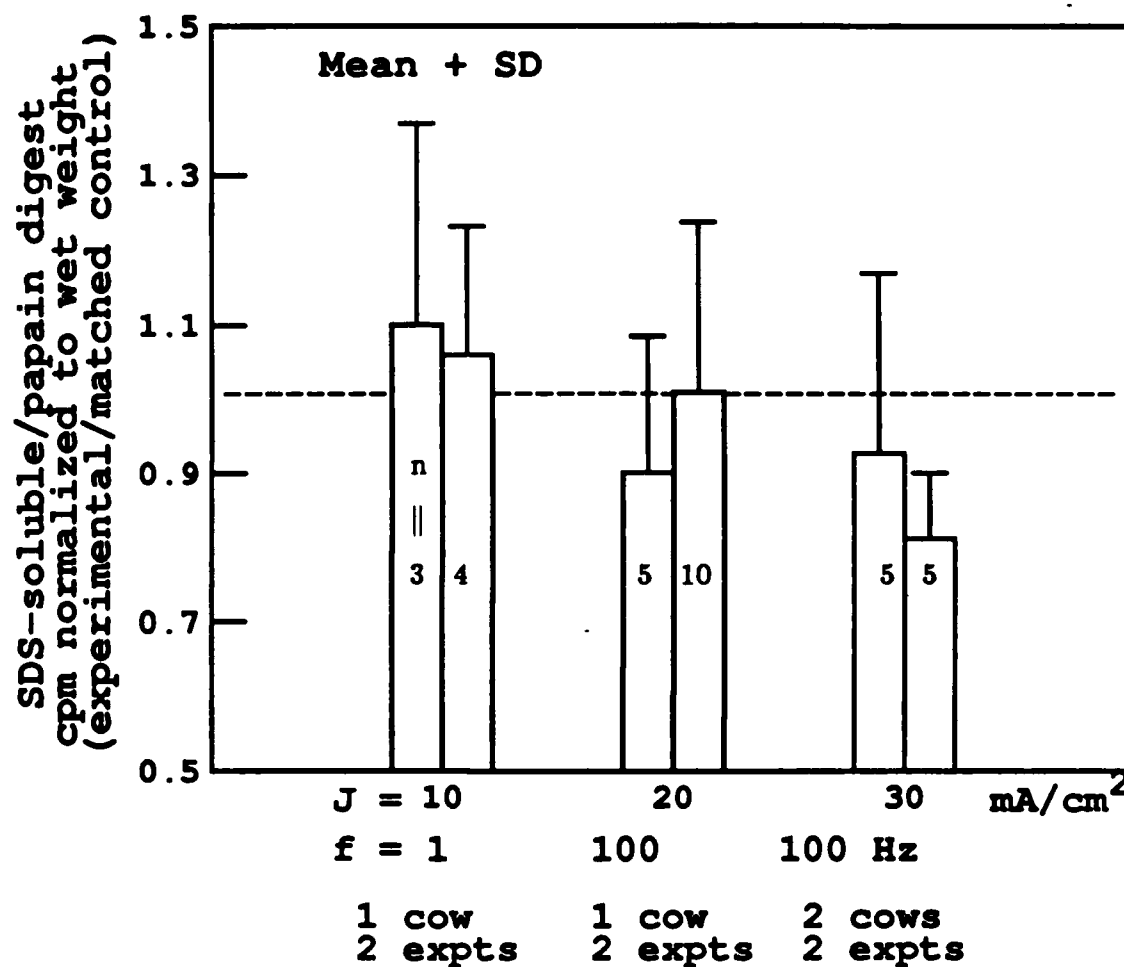


Figure 4.5: Variation by cow in the ratio of incorporation in the SDS extract relative to the papain digest for the same stimulation protocol (frequency and current density as labelled in the figure).

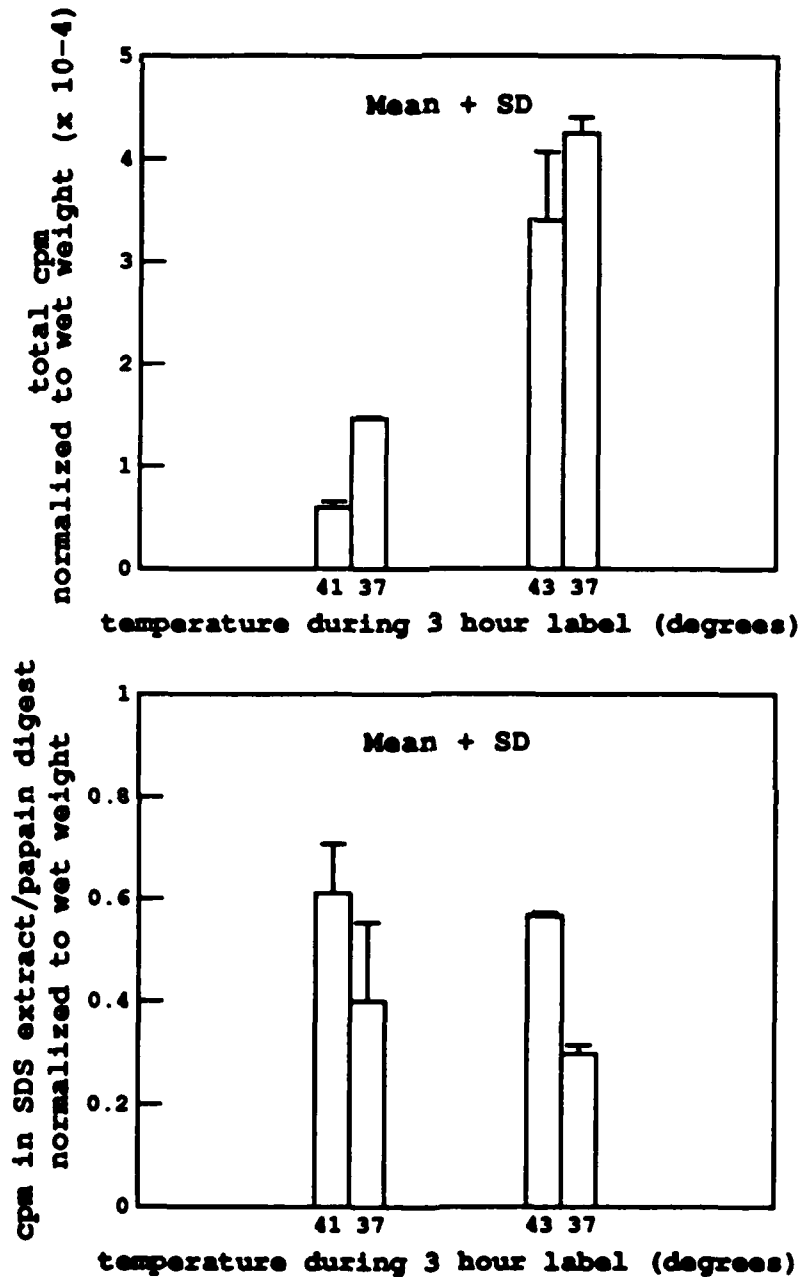


Figure 4.6: (A) Total incorporation or (B) SDS-soluble/papain digest incorporation for heat shocked and control cartilage plugs normalized to wet weight is plotted for two experiments. Both were done on day 7 after cutting. In the first, plugs were incubated and labelled at 41 or 37°C. In the second, using plugs from a different cow, plugs were incubated and labelled at 43 or 37°C. This data corresponds to the day 7 data plotted in Fig. 2.13 and 2.17.

also exhibit some frequency dependence, as discussed previously. However, further experiments would be required to substantiate either of these possible trends with frequency.

#### 4.3.6 Conclusion

In summary, the electrical stimulation experiments demonstrated that there was no stress response to current densities as high as  $30 \text{ mA/cm}^2$  for frequencies between 1 Hz and 10 kHz. There was however, an increase in total protein synthesis for current densities  $> 10 \text{ mA/cm}^2$ . Synthesis increased with current density and may have a maximum near 100 Hz - 1 kHz. It depended on the position on the joint surface from which the plugs were obtained and on the cow.

The lack of stress response indicates that the applied current densities do not act to stress cells by producing an accumulation of abnormal proteins. This is important in the context of biological hazards of fields in the environment, since it indicates that local current densities below  $30 \text{ mA/cm}^2$  do not stress cells for frequencies below 10 kHz. This current density is many orders of magnitude greater than fields induced within tissues by typical environmental sources within this frequency range.

The lack of stress response further served as a control to demonstrate that the increase in protein synthesis which was observed in response to electric fields was not due to ohmic heating (above  $39^\circ\text{C}$ ) and was not due to electrode reaction products. In fact, both an increase in temperature and electrode reaction products in the media resulted in a decrease in total protein synthesis (Fig. 4.6 and Table 3.1).

The increase observed in total protein synthesis is consistent with an increase in the overall level of synthesis (at least at 100 Hz and 1 kHz). The current densities which induced the increase in protein synthesis ( $> 10 \text{ mA/cm}^2$ ) are large relative to current densities in cartilage generated by mechanical loading (on the

order or less than  $1 \text{ mA/cm}^2$ ). However, the relatively large current densities required to induce a response do not imply that physiological current densities are not important. The variation between the cartilage specimens on the joint surface may obscure a response to lower current densities. Thus, the experiments suggest that physiologically generated current densities may play a role in biosynthetic control of chondrocytes.

The field-induced increase in incorporation into the papain digest, which includes proteins covalently bound to the matrix, is consistent both with an increase in overall protein synthesis and possibly with an increase in binding rate. Although the experiments involved short term exposures, the enhanced accumulation into the matrix might suggest a possible role for electric fields in growth and remodelling of cartilage. Furthermore, it is possible that incorporation in the papain digest might also increase following longer exposures to lower current densities.

In order to address the dual issues of the level of current density required to produce a biosynthetic change and the mechanism of interaction with the field, an experimental model such as chondrocytes cultured in agarose may be indicated. Chondrocytes cultured in agarose would provide more homogeneous specimens compared to cartilage tissue, and differences in electrical response between specimens might therefore be smaller. Such a model would be more easily manipulated, so the importance of factors which might govern the electrical response, such as basal synthetic level, cell density and matrix composition, could be assessed. Identifying the factors that govern field response might further aid in understanding the underlying mechanism by which fields act on the tissue.

## Appendix A

### Calculation of Field Magnitudes and Mechanisms

#### A.1 Calculation of Internal Field Magnitudes

##### A.1.1 Quasistatic Limit

In the quasistatic regime, the coupling between electric and magnetic fields can be neglected as far as calculation of internal fields is concerned. The upper frequency limit for the quasistatic approximation for the electric (or magnetic) case can be determined by first assuming a quasistatic solution for the internal electric (or magnetic) field, and then finding the perturbation in this solution due to electromagnetic coupling. The quasistatic solution is valid up to frequencies for which the magnitude of the perturbation approaches that of the assumed quasistatic solution. The EQS and MQS solutions are valid up to frequencies  $f \ll 1$  MHz for a spherical body of 0.5 m radius. This is below the long wavelength limit (wavelength  $\gg$  body dimension), as required.

In the EQS limit, the magnitude of the internal electric field induced in a spherical conducting body (conductivity  $\sigma$ , dielectric constant  $\epsilon$ ) by an external electric field  $E_o$  is

$$E_s = \frac{3\omega\epsilon_o E_o}{\sigma} \quad (\text{A.1})$$

In the MQS limit, the internal E field induced by an applied magnetic field that penetrates a conducting cylindrical body (conductivity  $\sigma$ , permeability  $\mu$ ) of radius  $r$  can be approximated as (H field orientation parallel to the axis):

$$E_\phi = \frac{\omega\mu_o H_o r}{2} = \frac{\omega E_o r}{2c} \quad (\text{A.2})$$

when the skin depth is long compared to body dimensions. The ratio of the magnitude of the magnetically induced internal E field (MQS model), to the E-induced

internal E field (EQS model) is,

$$\frac{E_{\phi MQS}}{E_{\phi EQS}} = \frac{\sigma r}{6} \left( \frac{\mu_o}{\epsilon_o} \right)^{1/2} \quad (A.3)$$

At these low frequencies, the conductivity,  $\sigma$ , is on the order of 1 mho/m, so this ratio is approximately,

$$\frac{E_{\phi MQS}}{E_{\phi EQS}} \approx 63 r \text{ (r in meters)} \quad (A.4)$$

In this limit the magnetic field produces the larger contribution to the total induced E field for r larger than a few centimeters.

The geometric intensification factors for prolate spheroid and ellipsoid models are larger than the corresponding factors in Eqn. A.1 and A.2 . However, the simplicity of these models emphasizes some salient features of the field distribution. These are the linear dependence on  $\omega$  (frequency), and the dependence on body dimension. It should be noted, however, that while the average MQS field magnitude is linear with the average body radius, a constriction in body dimensions (e.g. at the neck) will cause an intensification of induced current density proportional to the ratio of the areas, as demanded by continuity of current:

$$2\pi R_1^2 J_1 = 2\pi R_2^2 J_2 = \text{constant} \quad (A.5)$$

or, since  $J = \sigma E$ ,

$$E_2 = E_1 \left( \frac{R_1^2}{R_2^2} \right) \quad (A.6)$$

An additional geometric factor of about 10, should be used to obtain the correct magnitudes of the quasistatic fields induced using the simple models above, (so that the calculated absorbed power density assuming a uniform internal field is equal to the absorbed power density on which the standard is based).

In the high frequency, short wavelength limit, valid for frequencies  $\gg 1$  GHz



the field induced by an external E field parallel to the plane of the body is given by,

$$E_i = E_o e^{j(\omega t - kx)} e^{-z/d} \quad (\text{A.7})$$

where the thickness  $d = \sqrt{\frac{2}{\omega \mu_o \sigma}}$ . Since the tangential E field is continuous across the body boundary, this polarization represents the worst case: the polarization for which the magnitude of the internal field is largest for a given incident power density.

## A.2 Modulation of Transmembrane Potential

In Fig. 1.4, an idealized cell was modeled as a spherical shell representing the cell membrane. The intracellular space, cell membrane and extracellular matrix are described by uniform conductivities and dielectric constants as shown. The electric field induced inside, across, and in the local neighborhood of the cell due to a uniform external  $E$  field can be calculated for each of the three regions shown in Fig 1.4.

Models that treat the cell as an insulating sphere in a uniform field have been published previously [169,157,187] ] for the case of applied dc or very low frequency fields. The sinusoidal steady state treatment below is useful also for the case of higher frequencies (within the ANSI and ACGIH standards). Smith [189] developed a similar cylindrical model for a nerve axon for frequencies between 100 MHz and 100 GHz.

The initial transmembrane potential that exists in the absence of any applied field includes the electrical double layer potentials at the membrane interfaces. The effects of the applied field can be superposed to find the perturbation in the initial transmembrane potential. The induced transmembrane potential after application of an external field will vary with position around the periphery and is the difference between the induced potential evaluated at the external membrane surface and at the internal membrane surface at any angle  $\theta$ .

On macroscopic length scales larger than a Debye length (i.e.  $\delta > 1$  nm), there is no net charge in a uniformly conducting medium. Therefore, the electrical potential in each of the three bulk regions of Fig. 1.4 must satisfy Laplace's equation. Solution of this piecewise uniform electric field problem requires matching boundary conditions at each interface. This model addresses only macroscopic field quantities. Other molecular relaxation or interaction processes are not described by such a model. Charge relaxation (Maxwell-Wagner) at the inner and outer membrane surfaces is included and is fundamental to the form of the overall frequency response.

The potentials assumed for each region are:

Region I: external to cell

$$\phi_I = E_o r \cos \theta + \phi_e \frac{\cos \theta}{r^2} \quad (\text{A.8})$$

Region II: membrane

$$\phi_{II} = \phi_{m1} r \cos \theta + \phi_{m2} \frac{\cos \theta}{r^2} \quad (\text{A.9})$$

Region III: cell interior

$$\phi_{III} = \phi_c r \cos \theta \quad (\text{A.10})$$

The conditions which must be satisfied at each boundary are:

$r=R_e$ :

$$\phi_I = \phi_{II} \quad (\text{A.11})$$

$$[\sigma_e E_I]_r = -j\omega [\epsilon_e E_I - \epsilon_m E_{II}]_r \quad (\text{A.12})$$

$r=R_c$ :

$$\phi_{II} = \phi_{III} \quad (\text{A.13})$$

$$[\sigma_c E_{III}]_r = -j\omega [\epsilon_c E_{III} - \epsilon_m E_{II}]_r \quad (\text{A.14})$$

By substituting the assumed solutions for the potentials Eqn. A.8 to A.11, these boundary conditions are equivalent to a set of four equations in the four coefficients of the potential solutions. In terms of the potential coefficients, then, the induced transmembrane potential is,

$$\Delta V_m = \left( E_o R_e + \frac{\phi_e}{R_e^2} - \phi_c R_c \right) \cos \theta \quad (\text{A.15})$$

or equivalently,

$$\Delta V_m = \left\{ \phi_{m1} (R_e - R_c) + \phi_{m2} \left( \frac{1}{R_e^2} - \frac{1}{R_c^2} \right) \right\} \cos \theta \quad (\text{A.16})$$

With  $R_e = R_c + \delta$ , this reduces to,

$$\Delta V_m = \delta \left( \phi_{m1} - \frac{2\phi_{m2}}{R_c^3} \right) \quad (\text{A.17})$$

Simple expressions for the potentials in each region and the induced transmembrane potential can be found in three frequency regimes.

At the lowest frequencies, for which  $\frac{\omega\epsilon}{\sigma} \ll 1$ , and  $\frac{R_c \omega\epsilon}{\delta} \ll 1$ , the solution can be approximated by setting the normal external current to zero. In this case, the coefficients become,

$$\phi_e \approx \frac{E_o R_c^3}{2} \quad (\text{A.18})$$

$$\phi_c \approx 0 \quad (\text{A.19})$$

and the potential drop induced across the membrane is:

$$\Delta V_m \approx \frac{3}{2} E_o R_c \cos\theta \quad (\text{A.20})$$

This solution corresponds to the limit of instantaneous charge relaxation at the cell surface such that no external conduction currents penetrate the cell, and is the solution found in reference [169]. The factor of 1/2 has sometimes been neglected in more approximate models [157].

At slightly higher frequencies, as  $\frac{R_c \omega\epsilon_m}{\delta} \rightarrow 1$  but  $\frac{\omega\epsilon}{\sigma}$  still  $\ll 1$ , the potential is phase shifted relative to the imposed field, but the magnitude is not much different from the lower frequency limit. In this limit,

$$\phi_{m1} \approx \frac{E_o R_c}{2} \frac{R_c}{\delta} \left[ 1 + j \frac{3 R_c}{2} \left( \frac{\omega\epsilon_m}{\sigma} \right) \left( 1 + \frac{j\omega\epsilon}{\sigma} \right) \right]^{-1} \quad (\text{A.21})$$

$$\phi_{m2} \approx -\phi_{m1} R_c^3 \left[ 1 + \left( \frac{\omega}{\sigma} \right)^2 (\epsilon - \epsilon_m)(\epsilon + 2\epsilon_m) - \frac{j3\omega\epsilon_m}{\sigma} \right] \quad (\text{A.22})$$

In these expressions, terms in  $\frac{\delta}{R_c}$  were neglected relative to 1, and it was assumed

that  $\epsilon_c = \epsilon_s = \epsilon$  and  $\sigma_c = \sigma_s = \sigma$ . The transmembrane potential is then:

$$\Delta V_m \approx \frac{3}{2} E_o R_c \left[ 1 + j \frac{3}{2} \frac{R_c}{\delta} \left( \frac{\omega \epsilon_m}{\sigma} \right) \left( 1 + \frac{j \omega \epsilon}{\sigma} \right) \right]^{-1} \quad (\text{A.23})$$

In the special case  $\frac{\delta}{R_c} = \frac{3 \omega \epsilon_m}{2 \sigma}$ ,

$$\Delta V_m \approx \frac{3}{2} E_o R_c (1 - j) \quad (\text{A.24})$$

At high frequencies, where  $\sigma / \omega \epsilon \ll 1$ , charge relaxation does not have time to occur, and field-induced polarization charges at the interfaces determine the distribution of potential. Conduction is unimportant. The coefficients for the potentials in this case, are:

$$\phi_{m1} \approx \frac{\epsilon_c (\epsilon_c + 2 \epsilon_m)}{\epsilon_m (\epsilon_c + 2 \epsilon_c)} E_o \quad (\text{A.25})$$

$$\frac{\phi_{m2}}{R_c^3} \approx \frac{\epsilon_s (\epsilon_m - \epsilon_c)}{\epsilon_m (\epsilon_c + 2 \epsilon_c)} E_o \quad (\text{A.26})$$

The transmembrane potential is:

$$\Delta V_m \approx \delta \frac{\epsilon}{\epsilon_m} E_o \quad (\text{A.27})$$

again with  $\epsilon_c = \epsilon_s = \epsilon$ . Since at these high frequencies,  $\epsilon_m$  is not much different from  $\epsilon$ ,  $\Delta V_m$  is on the order of  $\delta E_o$ .

The transmembrane potential calculated using this spherical model can be compared to the transmembrane potential calculated for the different geometry of a cylindrical model of nerve fiber [189], for frequencies between 100 MHz and 100 GHz. Smith's model requires two solutions, one for an E field oriented along the cylinder axis, (the longitudinal solution), and one for a field perpendicular to the axis (the transverse solution).

Smith's solution for the cylinder model in the longitudinal orientation is an approximately uniform field equal to the magnitude of the imposed field in all

regions. This is to be expected provided the nodes in the myelin sheath have a negligible effects on the field, since tangential  $E$  is continuous across all boundaries and field decay is negligible over the dimensions of interest. The decay length for a 0.15M NaCl solution, even at the highest frequency (100 GHz) is greater than 300  $\mu\text{m}$ , which is large relative to the axon radius of 5  $\mu\text{m}$ . Thus, his solution predicts a negligible change in transmembrane potential. However, the infinite cylinder model cannot be used to calculate the transmembrane potential induced near the ends for this longitudinal orientation of the imposed field. This may be a significant failing, since as pointed out, the ends of an elongated cell or connected group of cells imply a large field intensification near that region [36].

The field solution for the transverse excitation for Smith's cylindrical cell model is equivalent to our solution for the spherical cell model, save for relative geometric factors of order unity. Both are Laplacian potential solutions with the constants set by boundary conditions at each interface.

Smith solved the general case with specific values of the electrical parameters. His calculations show small induced potentials over his entire frequency range even for a high field strength of 275 V/m within the tissue (larger than the field magnitudes induced by fields within the ANSI standard throughout this frequency range). The maximum change in transmembrane potential across the myelin sheath is calculated to be 1.6% of the thermal voltage, while across the membrane itself, it is only 0.13% of the thermal voltage. These small potentials are expected, since even at the lowest frequency considered ( $f = 100$  MHz),  $\omega\epsilon/\sigma = 0.4$  in the external tissue. The entire frequency range, therefore, lies above the frequencies for which a significant induced transmembrane potential is predicted based on the spherical shell model. The maximum change in potential which he calculated to occur at 1 GHz for the cylindrical model, is due primarily to the frequency dependence of the electrical characteristics he chose to represent each region of his model.

### A.3 Electrodiffusion: Modulation of Ionic Concentrations in the Extracellular Matrix and Within Cell Membrane

To model the extent of a field induced change in concentration, the initial concentration profile is approximated as linear in the absence of an externally applied field (Fig. 1.5):

$$c_i(x) = c_i(0) + [c_i(\delta) - c_i(0)] \quad (\text{A.28})$$

This profile might correspond to either an intramembrane or intratissue distribution. The gradient in concentration gives rise to a diffusion potential across the tissue or membrane. This potential is caused by the competition between the flux of ions due to diffusion and the flux of ions due to field driven migration, which must be balanced in equilibrium. An imposed field alters this balance between the diffusion and the migration fluxes. Biological tissues and membranes contain fixed charge groups. Within a charged tissue there is a much greater concentration of counter ions (ions with charge of sign opposite to the sign of the fixed membrane charge) than of co-ions, in order to preserve quasi-neutrality. This greater concentration of counter ions can shield out the self field of the fixed charge as far as transport of co-ions is concerned. As a result, the change in the minority carrier concentration due to an imposed field can be calculated to a good approximation without regard to the self induced field. The new concentration profile for the co-ion due to an imposed field is [75]

$$c_{co}(x) = \frac{[c(\delta) - c(0)] e^{E(z-\delta)/V_T} + c(0) - c(\delta) e^{-E\delta/V_T}}{1 - e^{-E\delta/V_T}} \quad (\text{A.29})$$

For small values of  $E\delta/V_T$ , this expression for the new concentration profile, when expanded to first order terms in  $E\delta/V_T$ , is identical to the initial linear concentration profile assumed in the absence of an imposed field. An expansion to second order terms in  $E\delta/V_T$  gives the dependence of the profile on the magnitude of the field,

for small values of the field.

$$c_{eo}(x) = \frac{c(\delta) - c(0) \left[ \left( \frac{x}{\delta} - 1 \right) + \frac{1}{2} \frac{E\delta}{V_T} \left( \frac{x}{\delta} - 1 \right)^2 \right] + c(\delta) \left( 1 - \frac{1}{2} \frac{E\delta}{V_T} \right)}{1 - \frac{1}{2} \frac{E\delta}{V_T}} \quad (\text{A.30})$$

The change in concentration due to the imposed field is,

$$\Delta c(x) = c(x)_E - c(x)_{E=0} \quad (\text{A.31})$$

or,

$$\Delta c(x) = \frac{1}{2} \frac{E\delta}{V_T} \left\{ [c(\delta) - c(0)] \frac{x}{\delta} \left( \frac{x}{\delta} - 1 \right) \right\} \quad (\text{A.32})$$

The difference between the field-free, and imposed field concentrations has a maximum at the midpoint,  $x/\delta = 0.5$ . Here, where the field induced change in concentration is greatest, the difference relative to the initial concentration for large initial gradients in concentration is:

$$\frac{\Delta c}{c_{E=0}} \approx \frac{1}{4} \frac{E\delta}{V_T} \quad (\text{A.33})$$

and for small initial gradients is:

$$\frac{\Delta c}{c_{E=0}} \approx \frac{1}{8} \frac{E\delta}{V_T} \left( \frac{c(\delta) - c(0)}{c(0)} \right) \quad (\text{A.34})$$



#### A.4 Rectification of Transmembrane Fluxes

The electrodiffusion model following assumes that the ion concentration within the membrane varies in quasi-equilibrium with the field. The interfacial concentrations, a fixed extracellular concentration, and an intracellular concentration which varies much more slowly than the concentration profile within the membrane, are treated as the fixed boundary conditions for finding the time varying intramembrane concentration. The solution for the membrane ion concentration profile can then be used to find the more slowly varying change in intracellular concentration.

The assumption of quasi-equilibrium is valid as long as the frequency is lower than the inverse of the electrodiffusion time (Chapter I). For the small nonthermal fields which are of particular interest ( $E\delta \ll 2\pi V_T$ ), the electrodiffusion time can be approximated by the diffusion time.

The electrodiffusion time for a membrane of thickness  $\delta=10$  nm, and an ion diffusion coefficient in the membrane of one fifth typical ion diffusion coefficients in water (as suggested by Pickard and Barsoum [163]), is approximately  $5 \cdot 10^{-8}$  s, which corresponds to 20 MHz. For frequencies much below 20 MHz, therefore, the ions respond fast enough that the concentration profile changes in response to the field in a quasi-instantaneous fashion. At frequencies near 20 MHz, a solution to the electrodiffusion problem in terms of spatial and temporal harmonics (modal expansion) is appropriate.

An estimate of the magnitude of the time average current which could be induced by an ac electric field by means of ion electrodiffusion is that magnitude induced by an external square wave field. The time average current density is

$$\langle J \rangle = \frac{1}{2T} \left( \int_0^T J(E) dt + \int_0^T J(-E) dt \right) \quad (\text{A.35})$$

where  $T$  is one half cycle.

The concentration profile and the current density within the membrane, can

be derived by solving the conservation of species equation subject to the boundary conditions on membrane concentrations (e.g. Appendix A.3). The form of the solution depends on the initial conditions assumed for the concentration or potential profile. Two classical models choose either an initial linear concentration profile, and consequently a nonlinear potential profile (the Planck model) [166], or a linear potential and consequently a nonlinear concentration profile (the Goldman model) [66]. It is useful to look at the nonlinear currents predicted by solving the electrodiffusion problem for the induced changes in membrane concentration with both sets of initial conditions. The different initial conditions apply depending on the type of ion(s) or membrane considered.

The assumption of a linear potential profile, or equivalently a spatially constant field within the membrane is consistent with Gauss' Law only as long as the net charge within the membrane is very small. This implies that for a single charged species in a neutral membrane, the membrane thickness must be less than or on the order of the Debye length [34]. In physiological saline, the Debye length is about a factor of ten smaller than typical cell membranes, so the constant field model is not appropriate for cell membrane transport of a single ion species. The constant field model may be appropriate for cell membrane models including several ions, such as  $\text{Na}^+$  and  $\text{K}^+$ , in an oppositely charged membrane, where the sum of the charge densities of the two free ion species are constant and equal to the membrane fixed charge density. A second example where the constant field approximation might be appropriate, is a model considering the transport of a co-ion, such as  $\text{Cl}^-$  in a negatively charged membrane, since in this case, the more numerous counter ions can adjust to preserve quasineutrality within the membrane (Appendix A.3).

In the remainder of this section, three solutions to the electrodiffusion problem are considered which may be relevant to the problem of rectification by a cell membrane or extracellular matrix. First the electrodiffusion problem is solved to find the concentration profile within the membrane. Second, this profile is used

to find the time average transmembrane current. Since the change in membrane ion concentration profile results in a non-zero time average current, membrane ion transport can be said to rectify the time-varying potential induced by the applied field. The concentration profile is calculated for a single ion species. However, the presence of other ion species may be implicitly assumed to set the initial field conditions.

The solutions differ only in that different terms of the conservation equation are held constant in space within the membrane. The first solution assumes a constant initial field (the resting potential is linear) as well as a constant applied field. The second solution assumes an initially linear concentration profile of a particular ion species (and so a space dependent field) but again a constant applied field. These two solutions might be relevant to rectification by  $K^+$  ion transport across the cell membrane, since the  $K^+$  ion is the major ion subject to passive transport across the membrane. The third solution assumes that (1) the initial field is small compared to the applied field, (2) the initial concentration profile is linear, and (3) the applied field is constant across the membrane. This solution is appropriate for considering the transport of the co-ion in a charged membrane through which there is a transport of both co-ions and numerous counter-ions. This might be a model for the transport of  $Cl^-$ , which is somewhat excluded by most cell membranes. However, since the membrane charge is located primarily at the membrane surface, this last model of excluded co-ion transport due to homogeneous membrane charge may be more appropriate for an extracellular matrix (such as cartilage).

In quasi steady state, (i.e. for frequencies below which the ion concentration profile can readjust in quasi-equilibrium with the change in field over each cycle), conservation of current is,

$$J = zF(\mu cE - D\frac{dc}{dx}) \quad (A.36)$$

where  $J$  is a constant to be determined by the boundary conditions on concentration

following integration of Eqn. A.36.

#### A.4.1 Constant Applied Field and Constant Initial Field

Conservation of current can be written to show the explicit spatial dependencies for a constant initial (linear resting potential) and applied field,  $E_o$  and  $E'$ ,

$$J = zF \left( \mu c(x) (E' + E_o) - D \frac{dc(x)}{dx} \right) \quad (\text{A.37})$$

The solution for  $c(x)$ , obtained by integrating Eqn. A.37, subject to the boundary conditions,  $c(0) = c_o$  and  $c(\delta) = c_\delta$  is:

$$c(x) = \frac{c_o (e^{kx} - e^{k\delta}) + c_\delta (1 - e^{kx})}{1 - e^{k\delta}} \quad (\text{A.38})$$

where  $k = \frac{E' + E_o}{V_T}$  as derived in Appendix A.3. The current density is then,

$$J = zF\mu E \left( \frac{c_\delta - c_o e^{k\delta}}{1 - e^{k\delta}} \right) \quad (\text{A.39})$$

In equilibrium, when no external field is applied ( $E' = 0$ ), the current density is zero, and the initial field  $E_o$  is related to the boundary concentration values by,

$$E_o \delta = \frac{RT}{F} \ln \frac{c_\delta}{c_o} \quad (\text{A.40})$$

The current induced by an applied field  $E'$  with an amplitude small relative to the equilibrium field, is obtained by expanding  $J$  of Eqn. A.39 in terms of

$$k'\delta = \frac{E'\delta}{V_T} \quad (\text{A.41})$$

for  $k'/k_o \gg 1$  where  $k' + k_o = E'/V_T + E_o/V_T = k$ . For a square wave applied field, the net  $J$  over one cycle is approximately given by the terms proportional to the

field squared in the expansion for J.

$$\langle J \rangle = zF\mu \frac{V_T}{\delta} \left( \frac{E'\delta}{V_T} \right)^2 \left( \frac{c_o c_\delta}{c_\delta - c_o} \right) \quad (\text{A.42})$$

#### A.4.2 A Constant Applied Field with a Linear Initial Concentration Profile

The second solution considered, that for an initial linear concentration profile, implies an initial field profile with spatial dependence. The statement of conservation of current, showing the explicit spatial dependence is,

$$J = zF \left( \mu c(x) [E' + E^o(x)] - D \frac{dc(x)}{dx} \right) \quad (\text{A.43})$$

The initial field  $E^o(x)$  can be related to the initial concentration,  $c^o(x)$ , by setting the current to zero in equilibrium, or,

$$E^o(x) = \frac{RT}{F} \frac{1}{c^o(x)} \frac{dc^o(x)}{dx} \quad (\text{A.44})$$

for positive ions. If the new concentration profile is a perturbation of the initial concentration profile,

$$c(x) = c^o(x) + c'(x) \quad (\text{A.45})$$

where

$$c^o(x) = (c_\delta - c_o) \frac{x}{\delta} + c_o, \quad (\text{A.46})$$

conservation of current can be rewritten for the change in concentration,  $c'(x)$ ,

$$J = zF \left( \mu \{c'(x)[E^o(x) + E'] + c^o(x)E'\} - D \frac{dc'(x)}{dx} \right) \quad (\text{A.47})$$

In Eqn. A.47 the equilibrium terms,

$$\mu c^o(x) E^o(x) - D \frac{dc^o(x)}{dx} = 0 \quad (\text{A.48})$$

have been eliminated. Solving Eqn. A.47 for  $c'(x)$  gives

$$\frac{c'(x)}{c^o(x)} = \left\{ [e^{k(1-x)} - e^{kx}] \left\{ \frac{\ln \frac{c_o}{c_\delta} + \sum_n \left( \frac{(-k\delta)^n}{n!} \right) \left( \frac{(c_o^n - c_\delta^n)}{(c_\delta - c_o)^n} \right)}{\ln \frac{c_\delta}{c_o} + \sum_n \left( \frac{(-k\delta)^n}{n!} \right) \left( \frac{c_o^n - c_\delta^n}{(c_\delta - c_o)^n} \right)} \right\} - (1 - e^{kx}) \right\} \quad (\text{A.49})$$

The corresponding current density induced by the applied field is then,

$$J = (c_\delta - c_o) \frac{e^{\left(-k\delta \frac{c_\delta}{c_\delta - c_o}\right)} - e^{\left(-k\delta \frac{c_o}{c_\delta - c_o}\right)}}{\ln \frac{c_\delta}{c_o} + \sum_n \left( \frac{(-k\delta)^n}{n!} \right) \left[ \left( \frac{c_o}{c_\delta - c_o} \right)^n - \left( \frac{c_\delta}{c_\delta - c_o} \right)^n \right]} \quad (\text{A.50})$$

Again, to find the time average current produced by a square wave, the current density of Eqn. A.50 is expanded for small applied fields. The quadratic terms of the expansion yield the time average  $J$ :

$$\langle J \rangle = (k\delta)^2 (c_\delta - c_o) e^{\left(\frac{-c_o}{c_\delta - c_o}\right)} \left( \frac{1 - \frac{1}{2} \ln \frac{c_\delta}{c_o}}{\left( \ln \frac{c_\delta}{c_o} \right)^2} \right) \quad (\text{A.51})$$

#### A.4.3 Constant Applied Field, Small Initial Field, and Linear Initial Concentration Profile

The last solution considered, that for an initial linear profile, a small initial intramembrane field, and a constant applied field is analogous to the first solution. The concentration profile is obtained from Eqn. A.30 with the substitution of  $E'$  for the quantity  $E_o + E'$ , or  $k\delta = E'\delta/V_T$ . The time average current is then,

$$\langle J \rangle = zF\mu \frac{V_T}{\delta} (c_\delta - c_o) \frac{(k'\delta)^2}{6} \quad (\text{A.52})$$

#### A.4.4 Magnitude of Field Induced Change

It has been shown above that an ac applied field can induce a time average current across a membrane. The time average may be significant if the current is associated with the transport of important ions (e.g.  $\text{Ca}^{++}$ ).

The  $\text{K}^+$  flux density out of a cell, can be calculated, as an example, using the solution for constant initial and applied field (Eqn. A.42) and the cell parameters:

$$c_i = 140 \text{ mM}, c_o = 5 \text{ mM (typical concentrations for } \text{K}^+ \text{ ions)}$$

$$k\delta = \frac{E\delta}{V_T} = 0.1$$

The flux density,  $\Gamma$  is  $1.3 \cdot 10^{-7}$  moles/cm<sup>2</sup> sec. While a cell might be able to compensate for the change in flux by active transport the additional metabolic energy required might be substantial (the leakage flux for  $\text{Na}^+$  is typically  $10^{-11}$  moles/cm<sup>2</sup> sec.)

If the cell did not compensate for ion loss by active pumping, the change in internal cell concentration can be calculated from,

$$\frac{\partial c}{\partial t} = -\nabla \cdot \Gamma_{r=a} \quad (\text{A.53})$$

where  $a$  is the radius of the cell. For a uniform concentration within the cell, this can be integrated to obtain

$$\frac{dc}{dt} = \frac{1}{a} \Gamma_a(\theta = 0), \quad (\text{A.54})$$

or for the solution with a constant initial field (Eqn. A.42),

$$\frac{dc}{dt} = \mu \frac{1}{a} \frac{V_T}{\delta} (k\delta)^2 \left( \frac{c_o c_i}{c_i - c_o} \right) \quad (\text{A.55})$$

At early times, or assuming that the change in internal concentration is small, the internal concentration is,

$$c_i(t) - c_i(0) = 50 c_o t \quad (\text{A.56})$$

for  $c_i \gg c_o$  and  $k\delta \approx 0.1$ . The ion flux, therefore, could lead to a rapid (on the

order of msec) change in internal concentration, in the absence of cell regulation.



### A.5 Modulation of Membrane Pore Size and Transport: Double Layer Repulsion

A simple one-dimensional model for estimating the effects of applied fields on the size of equivalent pores in a cell membrane or extracellular matrix represents the walls of the pore by two charged parallel plates, each of which supports an electrical double layer. A change in double layer repulsion force between the constituents of the pore walls (i.e., the parallel plate 'molecules'), due to an imposed field could lead to a change in the pore spacing. While a treatment of double layer repulsion between rod-like molecules in cylindrical geometry would be more accurate, this one-dimensional approach will still give a reasonable order-of-magnitude estimate of the interactions involved, and initial computations are simplified.

In equilibrium, the mechanical forces and chemical bonds that constrain the positions of the "plates" are such as to balance the electrical double layer repulsion force. An imposed field (Fig. A.1) could change the surface potential of the "plates". However, the potential gradient at the surface should not change. The potential gradient at the surface is proportional to the surface charge density. In the biological context of tissues at constant, physiological pH, it is a reasonable assumption that fixed charge groups attached to the pore walls will not change their ionization state, since this state is controlled predominantly by pH. As a result, the change in potential induced on the "plates" should be accompanied by a change in the plate spacing so as to maintain the same potential gradient at the interfaces.

In the limit of small surface potential, the potential gradient at the pore wall is given by,

$$\frac{d\phi}{dx} = \frac{\kappa\phi_0}{\cosh \frac{\kappa W}{2}} \quad (\text{A.57})$$

where  $\phi_0$  and  $W$  are the equilibrium surface potential and plate spacing with no field imposed, and  $1/\kappa$  is the Debye length. If an applied field were to change the

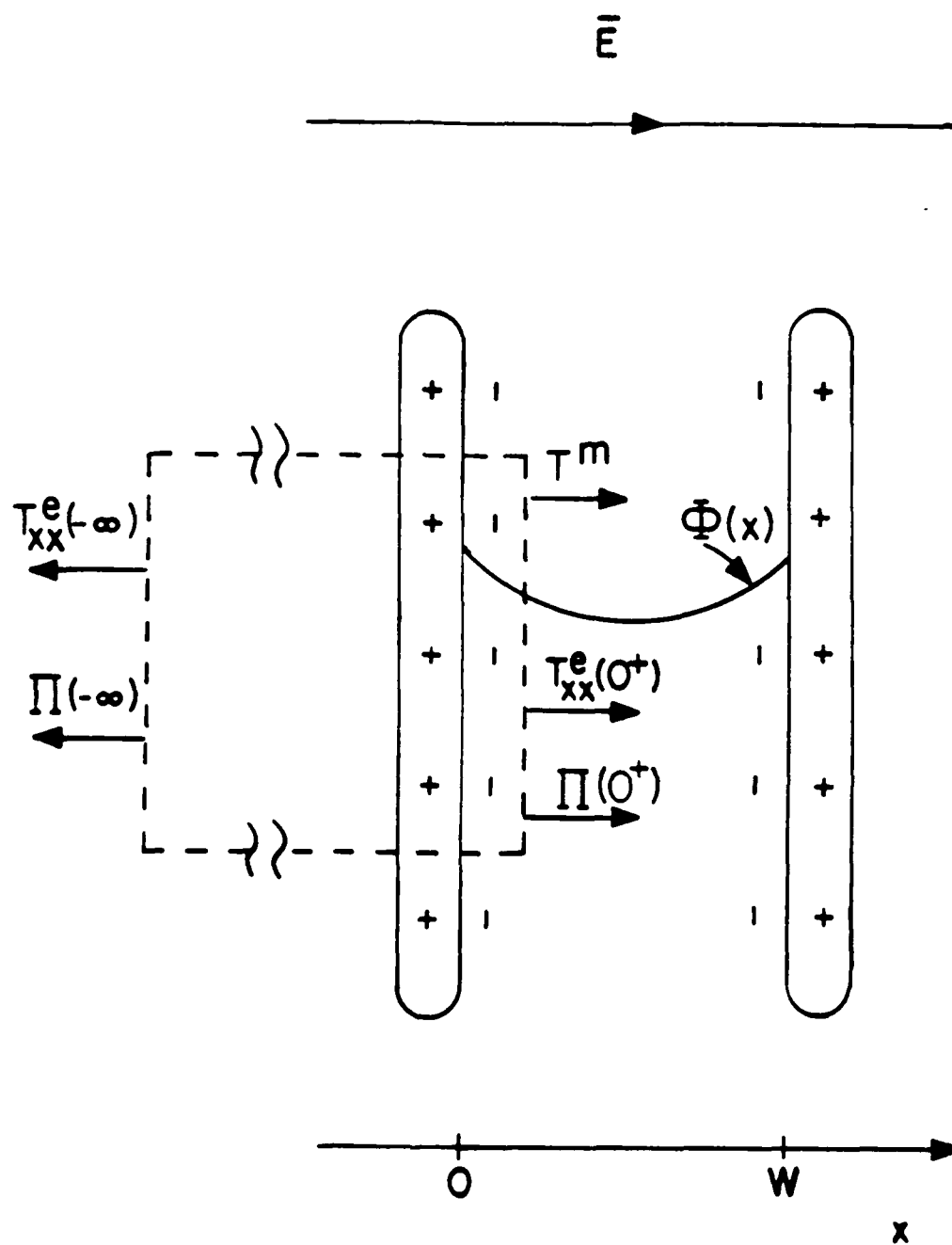


Figure A.1: A parallel plate model for modulation of pore spacing. The pore walls are represented by parallel plate "molecules".

surface potential to a new value,  $\phi'$ , the new gradient would be:

$$\frac{d\phi}{dx} = \frac{\kappa\phi'}{\cosh \frac{\kappa(W+\xi)}{2}} \quad (\text{A.58})$$

where  $\xi$  is the change in spacing between the plates. Since the gradient is held fixed, these two expressions must be equal, and the change in spacing can be specified in terms of the initial potential and the potential with field imposed:

$$\cosh \frac{\kappa(W+\xi)}{2} = \frac{\phi'}{\phi_0} \cosh \frac{\kappa W}{2} \quad (\text{A.59})$$

The new potential,  $\phi'$  can be obtained from a balance of the stresses acting on the plate before and after the field is imposed. For the surface defined in Fig. A.1, the sum of the stresses acting on the wall must be zero in equilibrium:

$$T^m + [T_{zz}^e(0^+) - T_{zz}^e(-\infty)] - [\Pi(0^+) - \Pi(-\infty)] = 0 \quad (\text{A.60})$$

where  $T^e$  is the electrical stress,  $T^m$ , the stress associated with mechanical constraints and bonds, and  $\Pi$  is the osmotic stress.

The electric stress far from the wall is zero initially, since for an isolated pore  $\frac{d\phi}{dx} \rightarrow 0$  as  $x \rightarrow -\infty$ . When the field,  $E_0$ , is imposed, the stress far from the wall becomes,

$$T_{zz}^e(-\infty) = \frac{\epsilon E_0^2}{2} \quad (\text{A.61})$$

In this one dimensional model, the initial equilibrium electrical stress at the wall ( $x=0^+$ ) due to double layer repulsion between the plates can be expressed in terms of the electric field at the surface of the pore wall:

$$T_{zz}^e = \frac{1}{2}\epsilon \left[ \frac{d\phi}{dx} \right]^2 = \frac{1}{2}\epsilon \left( \frac{\kappa\phi_0}{\cosh \frac{\kappa W}{2}} \right)^2 \quad (\text{A.62})$$

The second equality is strictly valid in the limit of small surface potential,  $\phi_0 \ll 1$ .

Since  $\frac{d\phi}{dx}$  is fixed, the stress at the pore wall is the same with a field imposed.

The osmotic pressure, can also be expressed in terms of the potential as,

$$\Pi(x) = 2RTc_0 \cosh \frac{zF\phi(x)}{RT} \quad (A.63)$$

As  $x \rightarrow -\infty$ , this becomes

$$\Pi(-\infty) = 2RTc_0 \quad (A.64)$$

while at  $x = 0^+$ , the osmotic pressure is:

$$\Pi(0^+) = 2RTc_0 \cosh \frac{zF\phi_0}{RT} \quad (A.65)$$

The new surface potential,  $\phi'$  can be found in terms of  $\phi_0$  and  $E_0$  by setting the sum of the stresses with no field imposed equal to the sum of the stresses with field imposed ( both sums being zero in equilibrium). To first order,  $T^m$  does not change significantly with the imposed field. That is, the change in 'plate' spacing due to the imposed field will adjust so as to minimize any change in  $T^m$ . In the limit of small surface potential, consistent with the development thus far, this stress balance reduces to:

$$\frac{\phi'}{\phi_0} = \left[ 1 + \frac{1}{2} \left( \frac{E_0}{\kappa\phi_0} \right)^2 \right]^{\frac{1}{2}} \quad (A.66)$$

For small values of  $E_0/\kappa\phi_0$ , this relation can be further approximated by,

$$\frac{\phi'}{\phi_0} = 1 + \left( \frac{E_0}{2\kappa\phi_0} \right)^2 \quad (A.67)$$

Then from Eqn. A.3, the equilibrium spacing between the plates with field imposed is obtained from:

$$\cosh \frac{\kappa W}{2} \left( 1 + \frac{\xi}{W} \right) = \left[ 1 - \left( \frac{E_0}{2\kappa\phi_0} \right)^2 \right] \cosh \frac{\kappa W}{2} \quad (A.68)$$

For small values of  $\kappa W$  ( $\kappa W \ll 1$ ), the change in spacing is,

$$\frac{\xi}{W} = \left( \frac{E_o}{2\kappa\phi_o} \right)^2 \left( \frac{1}{\kappa W} \right)^2 \quad (\text{A.69})$$

For large values of  $\kappa W$ , the change in spacing is,

$$\frac{\xi}{W} = \left( \frac{E_o}{2\kappa\phi_o} \right)^2 \frac{1}{\kappa W} \quad (\text{A.70})$$

The magnitude of the field  $E_o$  required to induce a 10% change in pore size (i.e.  $\xi/W = 0.1$ ) can be calculated from Eqn. A.69 and A.70 for different values of  $\kappa W$ . For physically reasonable values of the electric field inside the double layer ( $\kappa\phi_o \approx 10^6$  to  $10^7$  V/m), the calculated values of  $E_o$  can be compared to the field values that might be induced within the tissue space (e.g. fields induced by external fields within the ANSI standards). For most values of  $\kappa W$  the calculated  $E_o$  required to change the pore spacing by 10% is quite a bit larger than field values induced in the tissue space. The approximate nature of the model, however, implies that the numerical calculations are only order of magnitude. For small  $\kappa W$  and small  $\kappa\phi_o$  (i.e.  $\kappa W < 0.01$ , and  $\kappa\phi_o \approx 10^6$  V/m) the field calculated to change pore spacing by 10% are within an order of magnitude of the ANSI-derived internal field values. This might imply that this mechanism of pore modulation by the field should not be neglected. However, pore size is on the order of a Debye length ( $1/\kappa$ ) for pores within the cell membrane, so that  $\kappa W$  is of order one, and  $\kappa W > 1$  within the extracellular matrix. Thus the small values of  $\kappa W$ , for which induced fields might induce a 10% change in pore size are not physiological.

The above model is linear, and therefore is consistent with a sinusoidally varying pore size. However, since solute radius may be on the order of pore radius for many important solutes, the phenomenon of restricted diffusion can introduce a strong nonlinearity in the transport of solutes through a periodically varying pore structure. Diffusion times across a 5 nm membrane are  $10^{-8}$  to  $10^{-9}$  seconds.

Transport might therefore be affected as long as the pore can mechanically respond to the electrical force. Inertia at higher frequencies will eventually limit the pore's mechanical response. From the comparison of internal field values required, alone, changes in pore size would appear to be very small.

## **A.6 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis: SDS-PAGE**

SDS-PAGE is a recently developed, and now commonly used biochemical technique to separate and identify proteins by molecular weight [58]. It can be used in combination with other techniques (e.g. antibody or enzyme digestion assays) to identify specific proteins. The technique is based on field driven migration of charged macromolecules, such as proteins (as in Chapter I) in a polyacrylamide gel. SDS confers a constant negative charge to mass ratio to the proteins. The polyacrylamide gel provides a porous support for the separation which both limits diffusion and convection, and sieves the proteins during field driven migration.

Polyacrylamide gels are formed by polymerization of acrylamide and bis-acrylamide. Polymerization is initiated by TEMED and ammonium persulfate (in the gel system used here). The monomer acrylamide molecules form polymers which are randomly crosslinked by the bis-acrylamide, creating a web-like structure. The porosity of the gel depends on the concentrations of acrylamide, and on polymerization conditions (purity of reagents, concentration of initiators, temperature, oxygen, pH, and time) [16]. For well defined polymerization conditions, the porosity of the gel depends primarily on acrylamide concentrations. Highly reproducible gels give reproducible protein separations (as can be seen by comparing protein separation on the gels in chapters 2 and 3).

In the system used here, gels are cast between two parallel plates separated by thin (1.5 mm) spacers (Hoefer Sturdier Gel Electrophoresis Apparatus [97]). A 10% acrylamide separating gel, approximately 11 cm high is cast first. On top of the separating gel, a stacking gel (4% acrylamide) is cast using a comb mold, such that there are wells at the top of the stacking gel that are from a few mm to 1 cm wide and approximately 2 mm deep. The protein solutions are placed in these wells, and then electric fields are applied uniformly across the slab gel by means of wire electrodes immersed in an electrolyte solution contacting the top and bottom

ends of the gel. With the field applied, the proteins migrate in the gel, from top to bottom, and proteins are separated by the distance they migrate during field application. The field is removed when the smallest proteins (marked by a small molecular weight dye front) have reached the bottom of the gel. The gels described in chapter II and III were run under constant voltage. Voltage were chosen within the range 30-150 V. For a set voltage, the current varied during electrophoresis, due to changes in the conductivity of the gel (approximately 5-10 mA corresponded to a voltage of 30 V).

For SDS-PAGE, proteins are first boiled in a solution containing SDS, and (under reducing conditions)  $\beta$ -mercaptoethanol to charge the proteins (by SDS) and denature the proteins (that is, the proteins lose their secondary structure and become randomly coiled globular proteins).  $\beta$ -mercaptoethanol breaks disulfide linkages between protein subunits. SDS binds to the proteins in a constant ratio of SDS/unit weight of approximately 1 g SDS/1 g protein ([58]) or about 1 SDS molecule/3 peptide bonds [194]. The negatively charged SDS molecules thus confer a net negative charge to the protein that is proportional to its molecular weight. (With the exception of a few highly charged proteins, such as the histones, the native charge of most proteins is an order of magnitude smaller than the charge conferred by the SDS [194]. Glycoproteins, such as the extracellular proteins of the cartilage matrix, the collagens, fibronectin and chondronectin, do not bind SDS as well as simple proteins.

In free electrolyte, field driven motion of counter ions in the double layer of a spherical particle, (a simple model for a globular protein), is balanced by viscous sheer stresses associated with the fluid motion in the counter-ion double layer, and gives rise to particle motion governed by [145]

$$v = bE \quad (\text{A.71})$$



where the mobility  $b$  is

$$b = \frac{\epsilon \zeta}{\eta} \left\{ \frac{1}{1 + \frac{\epsilon \zeta \sigma_s}{\sigma \eta R}} \right\} \quad (\text{A.72})$$

where  $\sigma_s$  is the surface charge, and  $R$  is the radius of the particle.  $\zeta$  is the zeta potential which is proportional to the surface charge.

Thus, since the proteins in SDS gel sample buffer solution have a uniform charge to mass ratio, the protein mobility in solution would be approximately inversely proportional to molecular weight. However, pore dimensions in a polyacrylamide gel are on the order of protein dimensions, so that the gel serves as a sieve based on size. Acrylamide concentrations, which determine the porosity of the gel are chosen based on the molecular weights of the proteins of interest. (Here 10% acrylamide gels were used in order to resolve proteins in the 40-150 kD range, typical of the molecular weights of stress proteins.) In practice, the distance migrated by a protein is approximately inversely proportional to its log molecular weight. Proteins of known molecular weight, which are available commercially, are run at the same time as the samples of interest. By plotting the migration distance vs log molecular weight of the known molecular weight proteins as a calibration curve, the molecular weight of the sample proteins can be determined from their migration distance. A calibration curve to determine the molecular weights of the heat induced proteins, in the gel electrophoresis system used here, is shown in Fig. A.2

## Molecular weight calibration

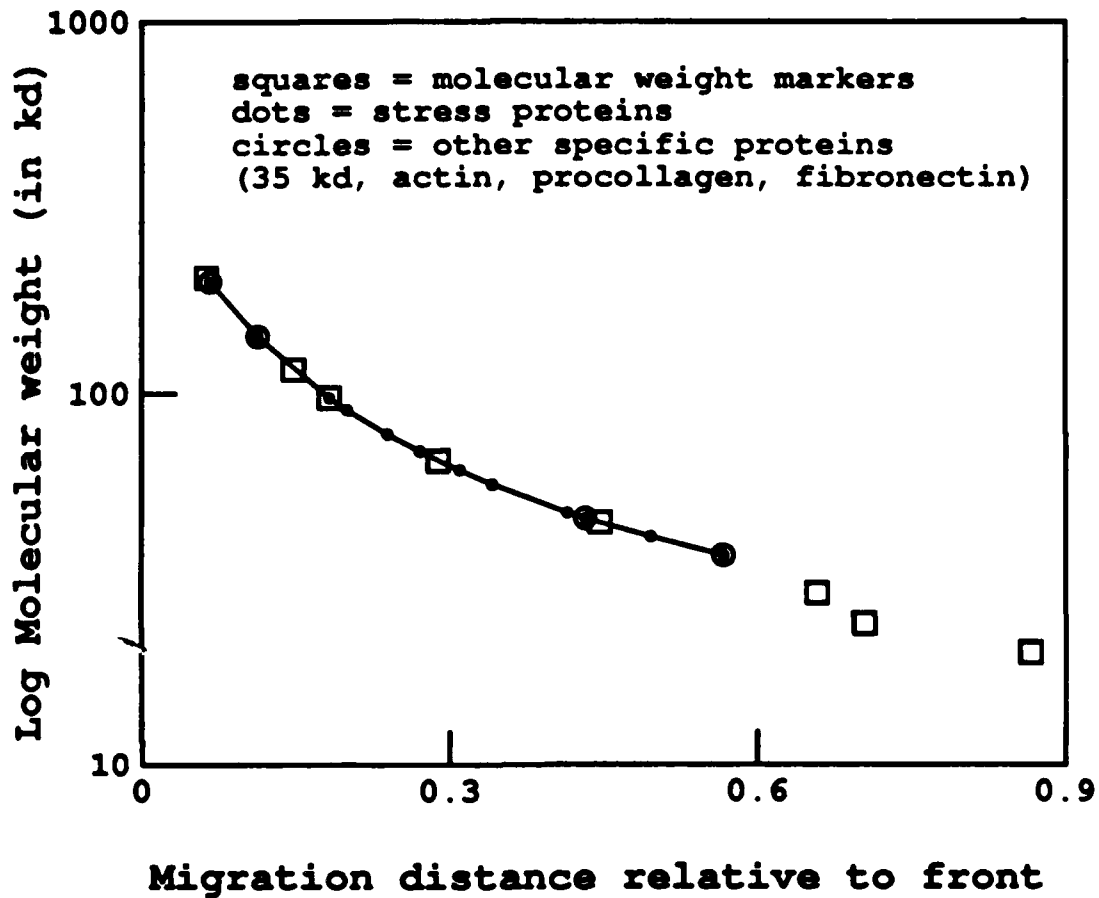


Figure A.2: Calibration curve for molecular weight determination. The migration distance travelled by standard molecular weight markers is plotted vs. log molecular weight (squares). Migration distance is defined by measurement of distance relative to front (e.g. relative to the dye front). The migration distance of the heat induced proteins are plotted (dots), and of 4 other proteins of interest (circles), and used to determine their molecular weights from the calibration curve.

## Appendix B

### Control Studies to Develop Experimental Protocol

#### B.1 Sample Preparation for SDS-PAGE

The two methods used to obtain samples for electrophoresis were: (a) initial isolation of cartilage cells from the tissue by means of collagenase digestion, followed by solubilization of intracellular proteins, or (b) solubilization of chondrocyte proteins *in situ* by boiling dry intact cartilage specimens in gel electrophoresis sample buffer.

The first method, following the method of Kubo and Treadwell [111,200], focuses on intracellular proteins. Plugs were first digested in 24 well culture dishes in a 5% CO<sub>2</sub> incubator for 8-10 hours with intermittent stirring, using 0.1% collagenase (w/v, Worthington Type II) in DMEM. The resulting chondrocyte suspension was spun down and resuspended twice in 1 ml HBSS. The chondrocytes were counted using a haemocytometer, spun down again and lysed in gel electrophoresis sample buffer (2% SDS, .0625M Tris, pH 6.8, 5%  $\beta$ -mercaptoethanol, 10% glycerol and 0.05% bromophenol blue). Two aliquots of the sample buffer were counted and radioactive counts were normalized to cell number for each sample. The remaining sample was run on a 10% polyacrylamide gel (Chapter II, Methods).

The second method enabled analysis of synthesis of both extracellular proteins incorporated into the extracellular matrix, and matrix proteins not yet incorporated into the extracellular matrix, as well as intracellular proteins. The intact cartilage plugs were first lyophilized, reweighed (dry weight) and then the dry plugs were boiled for 5 minutes in 100 to 150  $\mu$ l of gel electrophoresis sample buffer. The sample buffer solubilizes the chondrocyte proteins within the cartilage matrix, and permits the intracellular proteins and some of the non-incorporated extracellular proteins (e.g. proteoglycans) to diffuse out. Aliquots of the sample buffer were counted and radioactive counts were normalized to wet and dry weights. The remaining sample was run on a 10% polyacrylamide gel (as above).

Comparison of the protein bands corresponding to samples prepared by the two different extraction protocols showed few differences (Fig. B.1). Most importantly, the 70 kD stress protein appeared equally well on gel autoradiographs, using either protocol. Other protein bands were qualitatively similar, though not identical. Gels from samples obtained by the SDS extraction protocol had a high molecular weight protein band previously identified as procollagen [111,132,200]. This band is absent in gels from samples obtained by collagenase digestion. The latter gels had some additional very weak protein bands below 70 kD. These might be breakdown products due to the collagenase digestion.

Both methods yielded normalized radioactive counts that were similar from plug to plug, allowing for some variation in synthesis depending on the core position on the joint surface (Section B.8). Total radioactive counts for the samples obtained by collagenase digestion varied widely, due to non-uniform cell number. The non-uniformity in cell number appeared to be an artifact of the digestion. There was no apparent correlation between the number of cells obtained from a plug and the core position on the joint surface from which the plug was taken.

The collagenase digestion method, had several disadvantages compared to the SDS extraction method. The digestion process was time consuming and did not give uniform cell counts. Streaks occurred in the gel lanes (Fig. 2.3) perhaps due to the presence of fine residual collagen fibrils, or RNA and DNA. The gel buffer solubilization method was less time consuming, gave less variation in the total unnormalized radioactive counts per plug, and did not give rise to streaks in the gel lanes. Furthermore, subsequent papain digestion of the residual plug made it possible to study synthesis of proteins that have been incorporated into the extracellular matrix, as well as synthesis of non-incorporated extracellular and intracellular proteins.

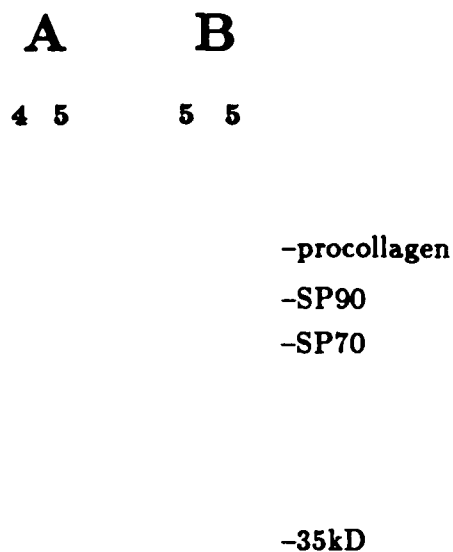


Figure B.1: Comparison of the protein bands obtained on gel electrophoresis of cartilage specimens following the two sample preparation protocols.

(A) Sample prepared following the collagenase digestion method

(B) Sample prepared following the SDS extraction method.

The number above each lane represents the day after tissue excision on which the specimens were labelled for 3 hours (e.g. 4 = day 4 after excision).

## **B.2 Resolution of Fluorography Compared to Autoradiography**

Gels exposed to X-ray film by fluorography are ready to develop several times faster than gels exposed by autoradiography. The resolution of the protein bands on the exposed film, however, is not as fine using fluorography. Fluorography has the additional disadvantage that the sensitivity of the film is not linear with total radioactivity in each band, especially for low radioactive counts. Linearity is improved by exposing the film at low temperatures (e.g.  $-70^{\circ}\text{C}$ ).

To compare the sensitivity of fluorography to autoradiography, in particular the resolution of the SP70 band, a gel was prepared and exposed, first following the protocol for autoradiography, and then for fluorography. A gel was chosen for which autoradiographic exposure had shown a clear SP70 protein band, but had not saturated the film exposure. The gel was then reswollen, and prepared following the protocol for fluorography.

For autoradiography, gels were stained with Coomassie Blue to visualize the marker proteins, destained, and reswollen in 7% acetic acid. The gels were then dried (BioRad Model 443 Slab Dryer), and placed in contact with Kodak X-omat AR film. For fluorography, gels were prepared following the method of Skinner and Griswold [188]. The gels were first stained and destained as described above, then soaked for 5 minutes in 10 volumes acetic acid, followed by 1.5 hours in 10 volumes 20% (w/v) PPO in acetic acid, and then 1/2 hour in water. Gels were dried at  $60^{\circ}\text{C}$  for 2-3 hours, and exposed to the Kodak film at  $-70^{\circ}\text{C}$ .

Although the resolution of the fluorograph was not as fine as the autoradiograph for the test gel, the SP70 band was equally clear on both. All other major protein bands were resolved using fluorographic techniques (Fig. B.2). Fluorography, therefore, was done routinely to obtain results as quickly as possible.

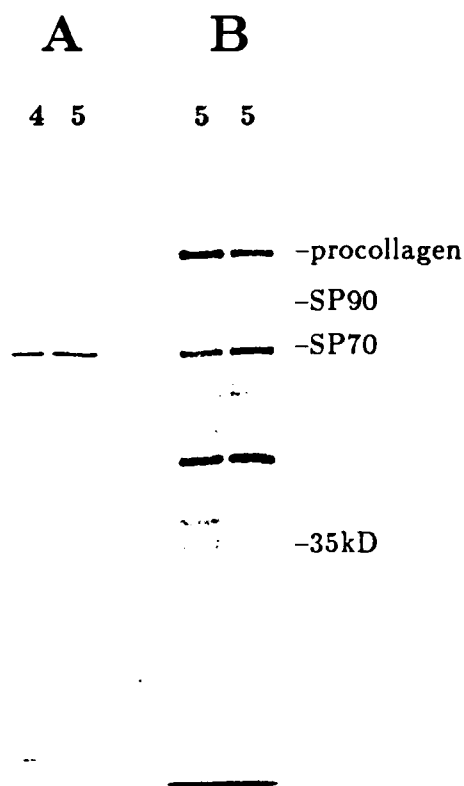


Figure B.1: Comparison of the protein bands obtained on gel electrophoresis of cartilage specimens following the two sample preparation protocols.  
(A) Sample prepared following the collagenase digestion method  
(B) Sample prepared following the gel sample buffer extraction method.  
The number above each lane represents the day after tissue excision on which the specimens were labelled for 3 hours (e.g. 4 = day 4 after excision).

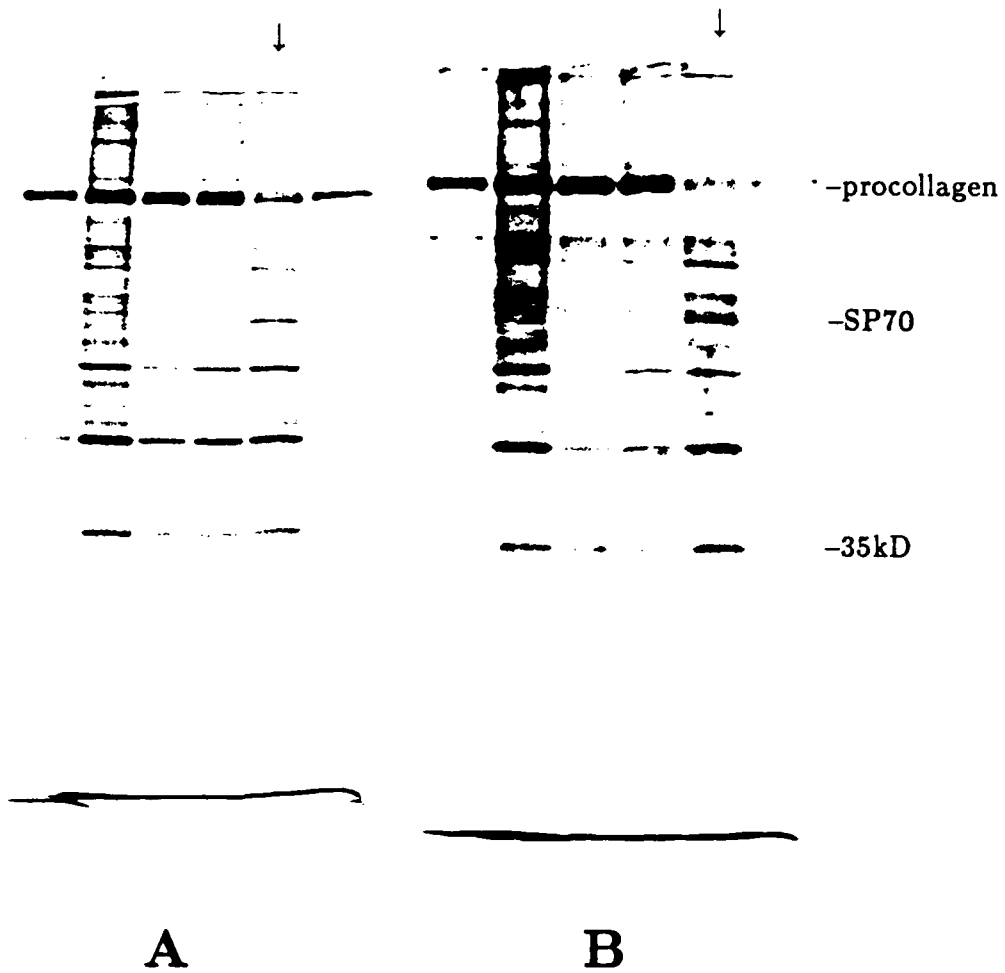


Figure B.2: Comparison of fluorography and autoradiography.  
(A) Gel exposed by autoradiography.  
(B) The same gel treated for fluorography (see text) and exposed.  
The SP70 and procollagen (135 kD) bands are labelled.



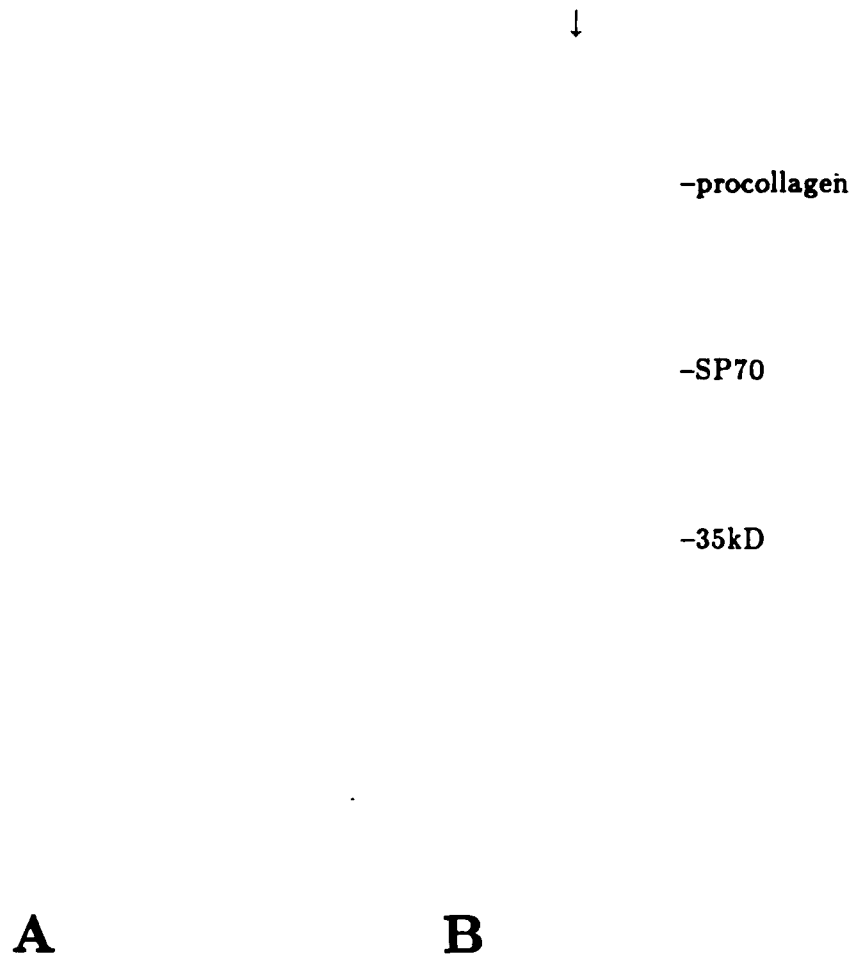


Figure B.2: Comparison of fluorography and autoradiography.  
(A) Gel exposed by autoradiography.  
(B) The same gel treated for fluorography (see text) and exposed.  
The SP70 and procollagen (135 kD) bands are labelled. The arrow points to the  
lane in which SP70 appeared.

### **B.3 Collagenase Digestion Assay for Procollagen**

Sensitivity to digestion by ultrapure collagenase was used to identify the procollagen band as described in Chapter II, Methods. Fig. B.3 shows the samples digested in collagenase with or without protease inhibitors, and two control samples from the same extract (with and without inhibitors).

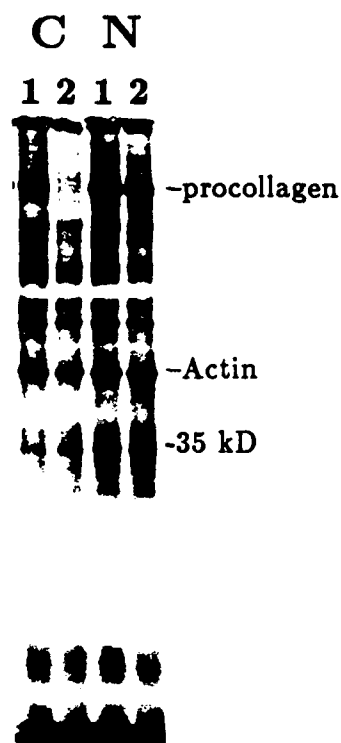


Figure B.3: Sensitivity to digestion by ultrapure collagenase was used to identify the procollagen band on a gel fluorograph (Chapter II, Methods) The 140 kd protein eliminated in the lanes corresponding to two digested samples, as shown in this gel fluorograph. The lanes correspond to samples that were digested by ultrapure collagenase (C) or not digested (N). The 140 kd protein (procollagen) is marked. Lanes 1 correspond to samples without protease inhibitors and lanes 2 to samples with inhibitors.

C N  
1 2 1 2

-procollagen

-Actin

-35 kD

Figure B.3: Sensitivity to digestion by ultrapure collagenase was used to identify the procollagen band on a gel fluorograph (Chapter II, Methods) The 140 kD protein was eliminated in the lanes corresponding to two digested samples, as shown in this gel fluorograph. The lanes correspond to samples that were digested by ultrapure collagenase (C) or not digested (N). The 140 kD protein (procollagen) is marked. Lanes 1 correspond to samples without protease inhibitors and lanes 2 to samples with inhibitors.

#### **B.4 Radiolabel Incorporation Dependence on Hot Methionine Concentration**

Incorporation of  $^{35}\text{S}$ -methionine was measured as a function of the concentration of  $^{35}\text{S}$ -methionine in the media. Cartilage specimens, 3mm in diameter and 1.6mm thick, were incubated at 37°C for 5 hours in DMEM with no methionine, and nominal concentrations of 5, 10, 20, or 30  $\mu\text{Ci/ml}$   $^{35}\text{S}$ -methionine. Following incubation, specimens were incubated for 1 hour in DMEM with cold methionine (2.0 mM), washed, weighed and processed as described in Chap II, Methods. Aliquots of the media used for incubation were counted, and used as the measure of concentrations of  $^{35}\text{S}$ -methionine in the labelling media.

Radiolabel incorporation was found to be nearly linear with the concentration of radiolabel in the media (Fig. B.4). This linear dependence is expected for incorporation that is proportional to the concentration of hot methionine relative to the total pool of methionine. These concentrations of hot methionine are in the nanomolar range, while the concentration of cold methionine in the media, due to diffusion of 2 mM methionine out of the plug liquid volume, is estimated to be on the order of 0.02 mM, for these 3mm diameter plugs (Appendix B.5). Thus the concentration of cold methionine is approximately equal to the total concentration of methionine, independent of the hot methionine concentration.

The linear dependence of incorporation on hot methionine concentration is useful in that the methionine concentration can be varied from experiment to experiment if required, and total incorporation can be normalized to the  $^{35}\text{S}$ -methionine concentration.

# cpm vs concentration of $^{35}\text{S}$ -methionine

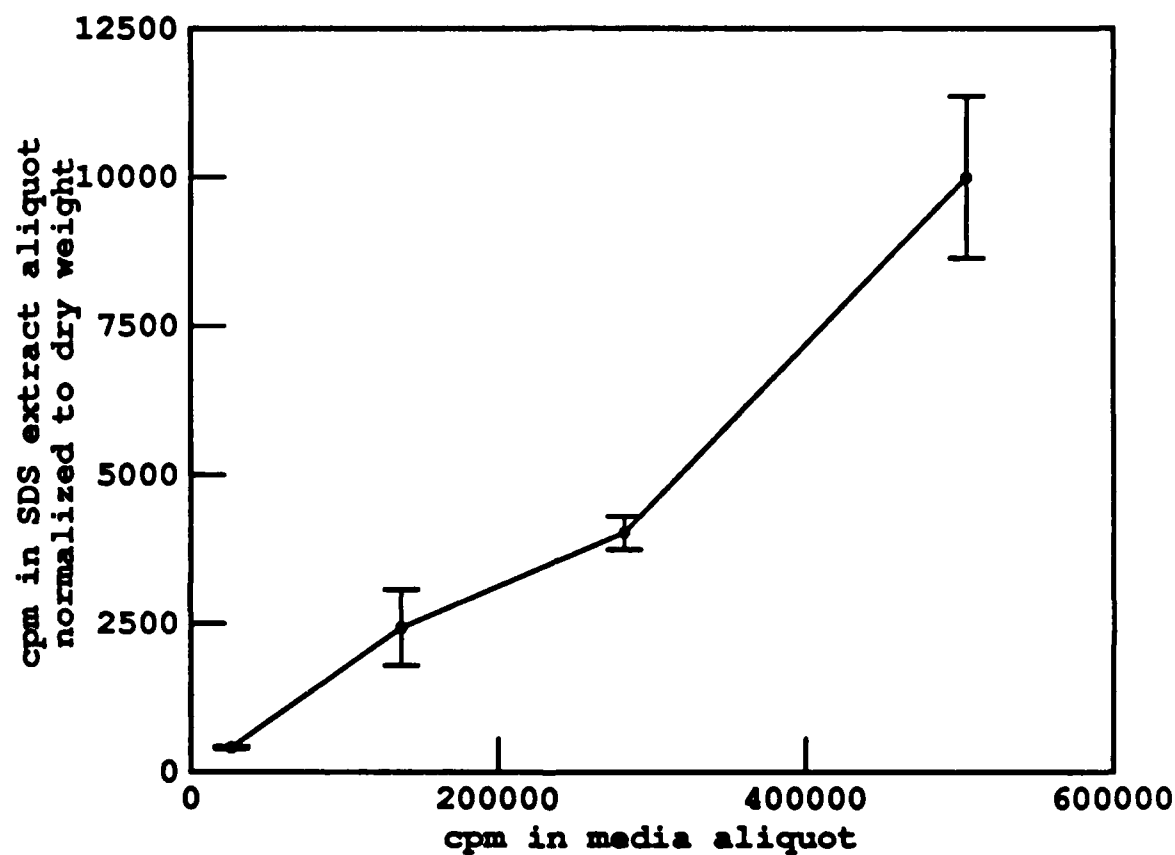


Figure B.4: Incorporation plotted as a function of hot methionine in the labelling media. Plugs (3 mm diameter, 1.6 mm thick) were labelled for 5 hours. The hot methionine concentration (x axis) is plotted as the cpm obtained by counting aliquots of the labelling media.

### B.5 Radiolabel Incorporation Dependence on Cold Methionine Concentration

To investigate the effect of added cold methionine concentration on radiolabel uptake, total  $^{35}\text{S}$ -methionine incorporation was measured as a function of cold methionine concentration between 0 mM (methionine free DMEM) and 2.0 mM (the concentration of methionine normally in DMEM). Total  $^{35}\text{S}$ -methionine incorporation was measured for plugs labelled for 6 hours in 0 mM (methionine-free DMEM), 0.001, 0.01, 0.1, 1.0 and 2.0 mM (normal DMEM) methionine in DMEM and incubated either at 37 or 43°C. Total incorporation was nearly independent of methionine concentration between 0 and 0.01 mM (Fig. B.5). Incorporation at 0.01 mM was slightly higher than at 0 or .001 mM, but the difference was not significant. Above 0.1 mM total incorporation decreased linearly with increasing concentration of added cold methionine (Fig. B.6).

The effect of changes in the concentration of cold methionine on total incorporation is as expected for radiolabel incorporation which is proportional to the ratio of hot to total methionine. Some cold methionine remains in the liquid volume of the plug and in residual media on surface of the plug when the incubation media is changed from the normal (2.0 mM methionine) to labelling media (0 mM methionine). If the methionine in the liquid volume of the plug diffuses into the labelling media, the effective concentration of total methionine in the labelling media is  $2 \text{ mM} \times (\text{plug liquid volume}/\text{media volume})$ . For a typical 1/4 inch diameter plug in 1 ml media, the effective concentration is approximately 0.06 mM (or for a 3 mm plug, 0.02 mM). Adding cold methionine in concentrations of less than 0.06 mM, therefore, does not change the effective cold methionine concentration in the labelling media, and consequently should have no effect on radiolabel uptake. In the control experiment, added concentrations of greater than 0.1 mM cold methionine decreased radiolabel incorporation, but less than 0.01 mM had no effect. These values bracket the estimated 0.06 mM.

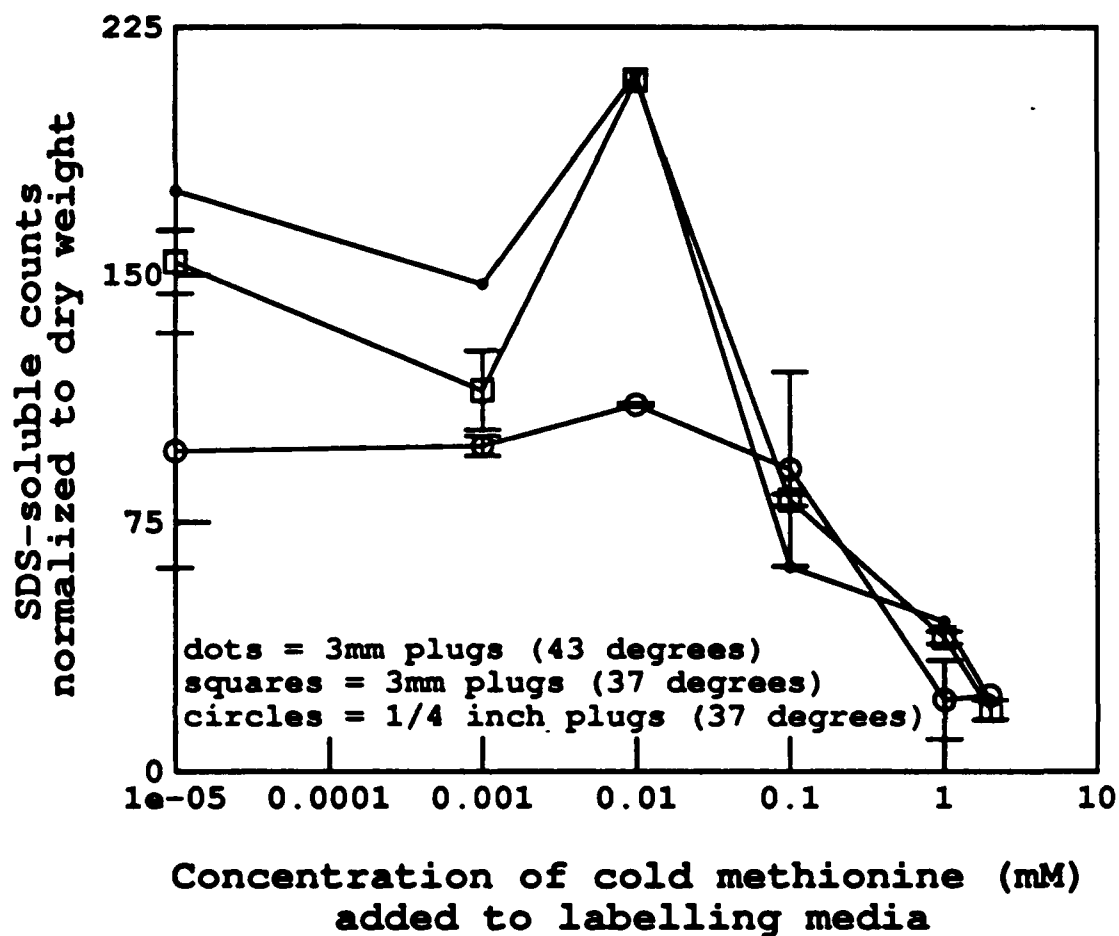


Figure B.5: Incorporation into SDS-soluble counts as a function of the concentration of methionine added to the labelling media. Normal culture media has a concentration on 2 mM methionine. Labelling media without methionine has a small amount of cold methionine from addition of 0.1%NS (approximately 1e-05). Intermediate concentrations were made from the appropriate combination of media with and media without methionine.



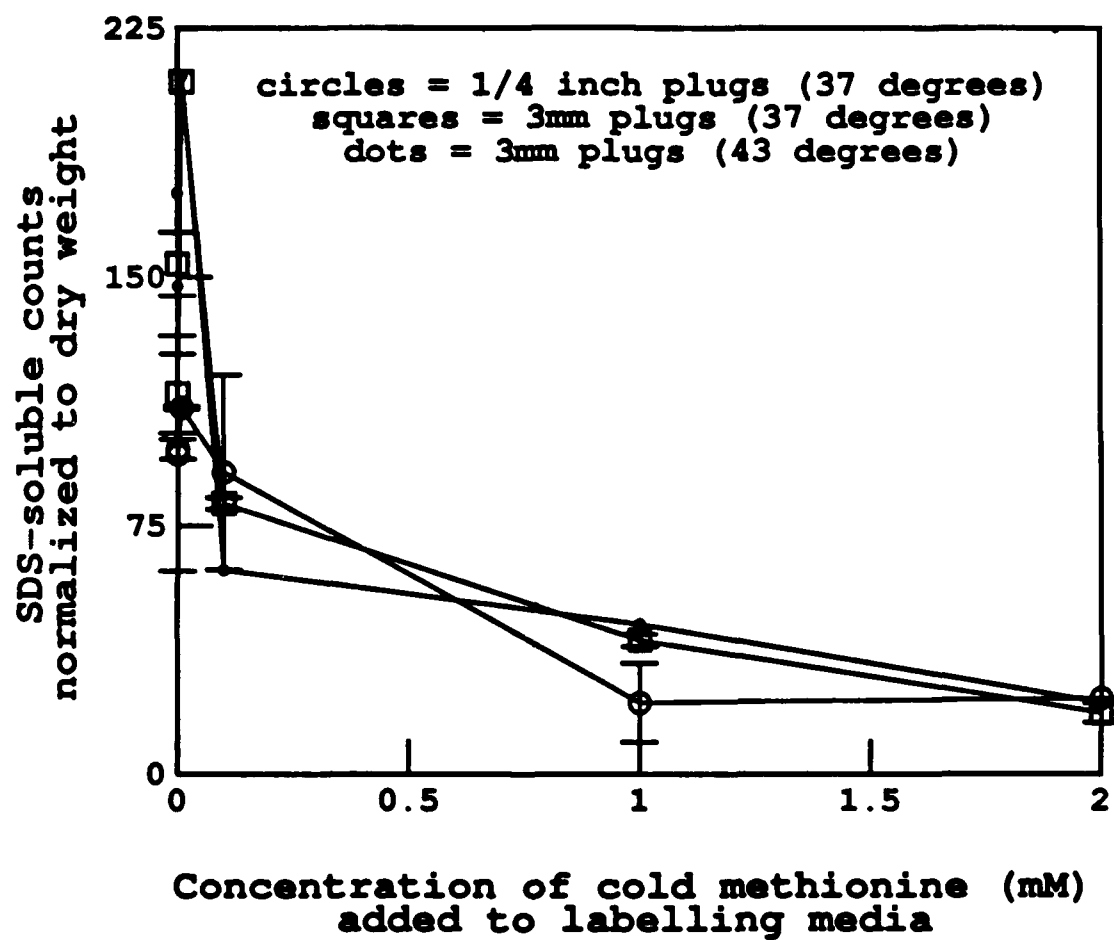


Figure B.6: Incorporation as a function of added cold methionine concentration (same data as in previous figure, Fig B.5, plotted on a linear scale).

For electrical experiments, therefore, in order to eliminate variation in incorporation due to variation in total methionine concentration, DMEM with 2.0 mM cold methionine (normal DMEM) was added to DMEM without methionine to obtain a concentration of 0.1 mM added cold methionine. This concentration is greater than both the calculated threshold above which the total methionine concentration depends primarily on the added cold methionine concentration, and the observed threshold concentration, above which plug to plug variation in incorporation (presumed to be due to variation in residual media volumes) was observed to decrease.

## B.6 Elimination of Free Label

To investigate the possibility that free  $^{35}\text{S}$ -methionine may be sequestered by chondrocytes during a six hour experimental period in media containing no added cold methionine, plugs were labelled for 6 hours and either washed immediately in HBSS or incubated at  $37^\circ\text{C}$  in media with cold methionine for 1/2 or 1 hour prior to washing. This procedure determines whether free label is present and will contribute artifactually to the total radioactivity in the gel sample buffer solution.

Total radioactivity of gel sample buffer extracts from plugs labelled with  $^{35}\text{S}$ -methionine in methionine free DMEM, followed by either no chase, 1/2 hour or 1 hour chase in DMEM with cold methionine was compared. TCA precipitation, followed by scintillation counting was also done on these samples. Paired plugs (from the 1st and 2nd mm of one position on the articular surface) were used to compare no chase to 1/2 hour chase, no chase to 1 hour chase and 1/2 hour to 1 hour chase. The counts for no chase samples were approximately twice the counts for either 1/2 hour or 1 hour chase samples (paired samples: mean counts for no chase/1/2 hour chase = 1.91, no chase/1 hour chase = 2.34). There was no statistically significant difference in counts for the 1/2 hour and 1 hour chase samples. On the gel fluorographs, no qualitative difference in the total amount of labelled protein was noted between lanes loaded with sample from plugs with and without chase. Counts obtained by several methods of TCA precipitation showed no apparent difference in total precipitable counts (e.g.  $^{35}\text{S}$ -methionine incorporated into protein) between no chase and either 1/2 hour or 1 hour chase.

The results of the chase experiment indicated that chondrocytes do sequester free label under experimental conditions, but that a half hour chase in DMEM with cold methionine at  $37^\circ\text{C}$  was sufficient to eliminate this effect. Consequently, all experiments routinely included a (minimum) half hour chase in cold methionine

after the labelling period.

## **B.7 Variation in Protein Synthesis with Serum Concentration (Literature Review)**

The choice of serum concentration in the media, and the frequency of media changes were based on some previous control studies performed in the continuum electromechanics laboratory [177,178,71], summarized below.

Gray assessed  $^3\text{H}$ -proline (protein synthesis) and  $^{35}\text{S}$ -sulfate (GAG synthesis) incorporation in minced epiphyseal cartilage tissue as a function of serum concentration and frequency of media changes. She labelled minced epiphyseal cartilage tissue each day for days 0-5 after tissue excision, using  $^3\text{H}$ -proline (protein synthesis) and  $^{35}\text{S}$ -sulfate (GAG synthesis) in media containing 0,0.1,1,2 or 5% NS. Subsequently, she labelled minced epiphyseal cartilage tissue maintained in media containing 0.1%NS daily, either with media changed every day or every two days, over a period of 2 weeks. She found that with daily media changes, incorporation of label was not sensitive to serum concentration, but that with less frequent media changes, incorporation was more sensitive to serum concentration. There was no significant difference in incorporation between tissue with daily media changes, and tissue with media changed every two days in DMEM supplemented with 0.1%NS.

Sah assessed  $^3\text{H}$ -proline incorporation (protein synthesis) and  $^{35}\text{S}$ -sulfate incorporation (GAG synthesis) in minced articular cartilage and whole articular cartilage plugs as a function of serum concentration. In all experiments, media was changed daily. Minced tissue maintained in 0,0.1,1 or 5%NS was labelled each day between day 0-6 after tissue excision [177]. Whole cartilage plugs, 3mm in diameter and 1 mm thick, maintained in 0.1 %NS were labelled each day between day 0-6 [177] and labelled on days 1-8 [178]. Whole cartilage plugs maintained in 1,3,5 or 20 %NS or 10 %FBS were labelled on day 0,1,5,9 and 14 after cutting [178]. Sulfate and proline incorporation in minced tissue was highest in tissue maintained in 1 or 5 %NS, lowest in 0 %NS, and intermediate at 0.1 %NS. In 0.1 % NS, incorporation was approximately constant over days 1-5, then dropped off on day 6. Both sulfate

and proline incorporation in cartilage plugs maintained in 0.1%NS was approximately constant between day 3 and 6, in the week long experiment. In the 8 day experiment (0.1 %NS) proline incorporation was approximately constant after day 3, while sulfate incorporation appeared to increase slightly between day 6-8. The ratio of proline to sulfate incorporation, therefore was constant between day 3-6, and decreased on day 6-8.

In the two week experiment in which different serum concentrations were used, both sulfate and proline incorporation were approximately the same in plugs maintained in either 1 or 3% NS, greater in 10% FBS, 20% NS, and greatest in 5%NS. As with previous experiments, incorporation increased in the first few days, and decreased to a steady state after 3 days. Following day 5, there was a further decrease in incorporation for plugs maintained in 1 or 3% NS.

In order that the cartilage specimens used in the electrical experiments be sensitive to field induced changes in incorporation in either direction, either an increase or a decrease, it is important that incorporation in control plugs (not electrically stimulated) be neither at a maximum or minimum. Further, for consistent conditions in a series of experiments, it is desirable that control incorporation does not change much between experiments. The experiments using 0.1%NS appear to indicate that maintaining plugs in 0.1%NS produces a rate of incorporation that does not change much after day 3, up to 8 days, and that this rate is not maximal or minimal (i.e. it is greater than incorporation at 0% serum and less than incorporation at higher concentrations of serum).

### **B.8 Variation in Protein Synthesis with Core Position (Literature Review)**

Protein and GAG synthesis (as assessed by incorporation of  $^3\text{H}$ -proline and  $^{35}\text{S}$ -sulfate, respectively) in untreated cartilage plugs was shown to be a function of the position on the joint surface from which plugs were taken [177]. Sah [177] calculated averages for the incorporation of each joint position (see Fig. 2.1 for nomenclature) normalized to average incorporation for the particular cow from which the cartilage plugs were obtained. He found that incorporation of both  $^3\text{H}$ -proline and  $^{35}\text{S}$ -sulfate was independent of the depth to 2 mm (i.e. whether the plug was 1st or 2nd mm from the surface), and independent of the joint—right or left. However, on average, incorporation in plugs from the medial facet was greater than in the lateral facet, and decreased from proximal to distal, more rapidly on the lateral than on the medial facet. Fig. B.7 (taken from [177], with permission) shows the variation of  $^3\text{H}$ -proline incorporation with position on both medial and lateral facets. There was a slight increase from position 1 to 2.

As a result of this study, throughout the electrical experiments, plugs were matched (experimental to control) according to position on the joint surface.

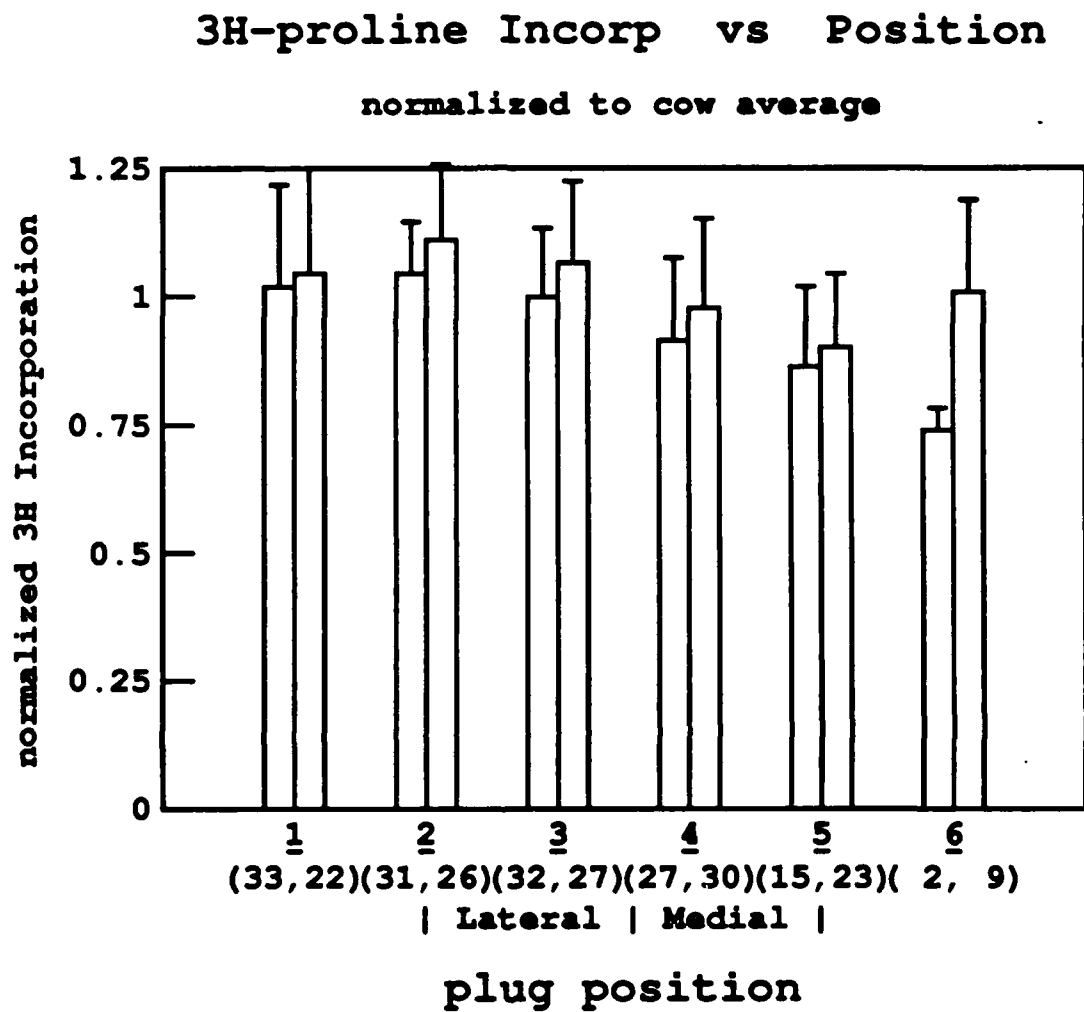


Figure B.7: Average  $^3\text{H}$ -proline incorporation, is plotted as a function of the position (1-6) on the joint surface from which plugs (1/4 inch diameter) were taken. Numbers represent the number of plugs in each average [177].



## Appendix C

### Electrical Stimulation Control and Pilot Studies

#### C.1 Protein Synthesis in the Electrical Stimulation Chamber

To check that the electrical stimulation chamber materials have no effect on protein synthesis, matched plugs were incubated and labelled simultaneously (using DMEM with no methionine) in the chamber (with and without field) and in 24 well culture dishes in the same incubator.

The protein band pattern was qualitatively the same for plugs incubated in the chamber and plugs incubated in the culture dish, assessed by examination of the gel autoradiograph. (It should be noted, however, that in two of thirteen subsequent electrical stimulation experiments, SP70 was synthesized by plugs in the control (unstimulated) chamber, as discussed below). However, the total amount of  $^{35}\text{S}$ -methionine incorporated by plugs in the chamber (as determined by counting the gel sample buffer) was somewhat (approximately 1.3 times) higher. The difference in total incorporation might be attributable to the difference in surface area available to diffusion of  $^{35}\text{S}$ -methionine. Incorporation in cartilage plugs has been shown to decrease with distance from the media-plug interface [Sah, unpublished data]. In the chamber, both faces of the plug were in contact with labelling media, while in the 24 well culture dish, only one face contacted the media. Differences in effective cold methionine concentration in the labelling media, due to diffusion of cold methionine out of the plug into different external liquid volumes (B.4) might also tend to increase incorporation in the chamber, since the media volume per plug is approximately 3.5 times the media volume per plug in the dish, so that the cold concentration of methionine in the chamber would be higher if diffusion of cold methionine out of the plug was the same in the two configurations. Thus, there was no important difference in protein synthesis in paired plugs in the electrical stimulation chamber (no field applied) compared to those in 24 well culture dishes.

Although the initial control experiment showed the electrical stimulation chamber materials did not affect protein synthesis, SP70 was produced by plugs in the control chamber in two subsequent electrical experiments. Subsequent to improvements in procedure (detailed below), no SP70 was observed on gel fluorographs obtained from plugs incubated in the control chamber.

The SP70 synthesis might have been the result of chamber contamination, insufficient cooling of the chamber after autoclaving, viral infection (no bacterial infection occurred) or SP70 synthesis in the absence of external stimulation. Chamber contamination might have resulted, for example, from rust on the clip lead connectors which might lodge in the holders during washing, or incomplete washing. The procedures for chamber preparation were subsequently modified. Viral infection is unlikely to be the cause of SP70 synthesis by a single plug (as in one of the two experiments), but it cannot be ruled out when all plugs produce SP70 (as in the second of the two experiments). Naturally occurring SP70 synthesis by chondrocytes from osteoarthritic adult articular cartilage has been observed [111]. Although osteoarthritis is unlikely in two week old calves, some other type of physical trauma or cartilage degeneration might have stimulated the chondrocytes to synthesize SP70 in vivo. Hightower and White [94,92] suggested that stress protein synthesis may be a response to tissue wounding and trauma.

As a result of these electrical experiments in which SP70 was synthesized in control chambers, all subsequent experiments routinely included a culture dish control (at both 37 and 43°C). The culture dish control at 37°C controlled for SP70 synthesis in control chamber or electrically stimulated plugs due to viral contamination prior to placing the plugs in the chamber, and for naturally occurring SP70 synthesis by the chondrocytes of a particular cow joint. The culture dish control at 43°C served to confirm that the chondrocytes of a particular cow joint were able to synthesize SP70.

In an effort to eliminate problems with contamination, a protocol for cham-

ber cleaning was established (including a minimum 3 hour soak in detergent, scrub, 3 rinses in tap water, 3 in distilled, followed by a 1 hour soak in distilled). To avoid insufficient cooling after autoclaving, the chamber was autoclaved the day before (when possible), or a minimum of 3 hours before an experiment. Further, all electrode connections were replaced by platinum wire to eliminate non-platinum metal contamination in the wash. Agar salt bridges were added to separate the cartilage plugs from the electrodes. Subsequent to the changes in chamber preparation no SP70 synthesis in the control chamber was observed.

## C.2 Casting and Control for Toxicity of Agar Bridges

Agar plugs were cast in a cylindrical tube teflon holder. The original holders were 1 cm long, later holders (those used throughout the electrical experiments) were 1.5 cm long, 3/8 inch outer diameter, and 3 mm inner diameter. The holders were placed in a teflon mold for casting that consisted of a block of teflon with 3/8 inch diameter holes milled to a depth of approximately 1.2 cm. The agar solution (2% (w/v) agar in DMEM) was autoclaved in a small media bottle, at the same time as the mold and pipets. While all were still hot, the agar solution was pipetted into the holders, and then allowed to cool at room temperature under the hood.

Three experiments were done to test that the agar bridges would not induce a stress response or alter protein synthesis. In the first cartilage rings (the remainder of the cartilage core after plugs were punched out) were incubated and labelled for 6 hours at 37°C, in 24 well culture dishes either in media containing the agar plug in its holder (1 ring and 1 holder/well), or in media without agar (control). In the second, rings were incubated and labelled for 6 hours, either in media in which an agar plugs in its holder had previously been incubated for 12 hours (the duration of an electrical experiment) and still containing the holder during the labelling period, or in media without agar. In these two experiments, agar plugs were cast using DMEM (with 2.0 mM methionine). In the third, plugs were incubated and labelled for 6 hours either in media in which an agar plug (pushed out of the holder) had previously been incubated for 12 hours, and still containing the agar plug, or in media without agar. In this last experiment, agar plugs were cast using DMEM with no methionine.

No SP70 synthesis was induced in plugs incubated with the agar plug and holder in any experiment.

Total <sup>35</sup>S-methionine incorporation into SDS soluble protein in cartilage rings incubated with agar decreased relative to incorporation in matched controls (1st experiment: mean=0.42±0.03, 2nd experiment: mean=0.69±0.34). However, this

decrease is most likely not an effect of the agar, but due to decreased diffusion of label into the plug due to contact between the solid holder and cartilage ring surface, and more importantly to the possible more than doubling of cold methionine concentration due to diffusion of cold methionine out of the agar plug (B.4). This explanation for the decrease in incorporation was supported by the results of the third experiment, in which the agar plugs were removed from the holder, and DMEM without methionine was used to cast the agar plugs.

In the third experiment, there was no significant difference in incorporation between cartilage plugs incubated with agar, and control plugs (in terms of total cpm/wet weight for  $^{35}\text{S}$ -methionine: experimental mean =  $31.16 \pm 7.65$ , control mean =  $27.52 \pm 2.57$ , and for  $^3\text{H}$ -proline: experimental mean =  $31.55 \pm 6.46$ , control mean =  $34.64 \pm 9.99$ ).

Taken together, the results indicated that the agar plug did not induce a stress response or a change in total protein synthesis, provided that the agar plugs were cast using DMEM with no methionine.

### **C.3 Pilot Electrical Stimulation Studies**

#### **C.3.1 Materials and Methods**

##### **Specimen preparation**

Cartilage specimens were prepared following the protocol described previously. For the studies reported here, both 1/4 inch diameter and 1 mm thick, and 3 mm diameter and 2 mm thick plugs were used. Specimens were maintained in DMEM supplemented with 0.1%NS (and antibiotics for the first 2 days). Media was changed every two days.

##### **Stimulation chambers**

Two electrical experiments used an early version of a teflon exposure chamber that is suitable for low current densities. All others were done using a new chamber that was designed and built so that the media could be cooled by an external circulating water bath.

The earlier chamber has been described previously [71]. It consisted of a block of teflon into which wells are cut. The wells were connected by tygon tubing prior to each experiment. Cylindrical cartilage holders fit tightly into a depression within each well, so that current was constrained to pass through the cartilage samples. Stimulation was via flat rectangular electrodes at each end of the row of wells.

The second chamber consisted of a pair of cylindrical teflon tubes filled with media (Fig. 3.1). Platinum electrodes capped each end of each tube. Each teflon tube was supported in a rectangular pan by six aluminum and stainless steel braces. Plugs (6 control and 6 experimental) were placed in concentric cylindrical teflon holders. The holders were then individually inserted into slots in the tubes. Electrical stimulation was imposed by means of the cylindrical platinum electrodes at each end of the tube and current is applied to the six specimens in series. The

tubes could be half submerged in a waterbath to maintain a constant temperature at high current densities, if required.

Chambers were prepared by soaking in a detergent solution, scrubbing and rinsing, and then assembled and autoclaved.

### **<sup>35</sup>S-methionine Incorporation During Electrical Stimulation**

Plugs were labelled in methionine free DMEM supplemented with 0.1 % NuSerum (NS) with 5 $\mu$ Ci/ml <sup>35</sup>S-methionine for 3,6 or 12 hours either during or immediately following electrical stimulation. For electrical stimulation, the cartilage plugs were first inserted into the cartilage holders with forceps, and the holders are then placed in the chamber. Control plugs were matched to experimental plugs by core position on the joint surface. Media, with or without label, was then pipetted into the chamber so that the cartilage plugs were completely covered. The resistance between the electrodes was measured for the experimental chamber to check for the presence of bubbles in the media, especially in the cartilage holders. The media filled chamber was then placed in the incubator (37°C, 5% CO<sub>2</sub>) and the electrodes were connected to the power supply.

After stimulation for the designated time at a given frequency and current density, the chamber was removed from the incubator. Media from all positions in the chamber was plated on blood agar plates (Brucella, Horse Serum), and the plates were placed in the bacteriological incubator for three days to check for bacterial growth. Following plating, the media was aspirated out of the chamber. The cartilage holders were removed, and the cartilage plugs were pushed gently out of the holders into a 24 well dish. Cartilage plugs that were labelled during stimulation were then washed, weighed and processed as described earlier. Cartilage plugs that were not labelled were incubated in labelling media for a designated period of time, and then washed, weighed and processed.

### C.3.2 Electrical Stimulation Experiments

A total of 14 electrical stimulation experiments were done (2 at 1 Hz, 10 at 10 Hz, 1 at 100 Hz and 1 at 1 kHz). With the exception of the first two experiments, stimulation was done on or after day 3 after cutting (based on early control studies, showing that total protein synthesis reached a steady state after 3 days).

Cartilage plugs (a total of 8 control and 8 experimental for each experiment) were subjected to a current density of 2 mA/cm<sup>2</sup> at 10 and 100 Hz, using the first version chamber. Plugs were stimulated and labelled simultaneously for 6 hours on days 1 and 2 after cutting.

With the second chamber, plugs were stimulated at low current densities (less than 10 mA/cm<sup>2</sup>) at frequencies of 1 and 10 Hz, and 1 kHz, and at high current densities (greater than 25 mA/cm<sup>2</sup>) at 10 and 100Hz. With a current density of 1 mA/cm<sup>2</sup> at 1 Hz, plugs were (1) stimulated and labelled simultaneously for 6 hours, and (2) stimulated for 12 hours and then labelled for 3 hours. At 10 Hz and current densities of 2 mA/cm<sup>2</sup>, plugs were (1) stimulated and labelled simultaneously for 6 hours (4 experiments), (2) stimulated for 6 hours and then labelled for 3 hours, and (3) stimulated for 3 hours and then labelled for 3 hours. Also at 10 Hz, plugs were stimulated and labelled for 6 hours using current densities of 3, 3.5, and 4 mA/cm<sup>2</sup> (1/4 inch plugs) and 9 mA/cm<sup>2</sup> (3mm plugs). At 1 kHz and 2 mA/cm<sup>2</sup>, plugs were stimulated and labelled for 6 hours.

### C.3.3 Results

Neither experiment in the first chamber (2 mA/cm<sup>2</sup> at 10 and 100 Hz) showed any apparent difference between control and experimental plugs in total incorporation of label or relative incorporation of label into papain digested sample fractions. No stress protein synthesis was observed on the gel autoradiographs.

In a single experiment in the second chamber, with no agar plugs between the cartilage specimens and the electrodes, electrically stimulated plugs (2mA/cm<sup>2</sup>,



10 Hz) synthesized SP70, as seen by gel autoradiography, while those in the control (unstimulated) chamber, with one exception, did not. The samples from several experiments done in the second chamber (in 4 experiments at 10 Hz at 2, and 3 and 9 mA/cm<sup>2</sup>, and in 1 experiment at 1 Hz, 1 mA/cm<sup>2</sup>) did not synthesize SP70 or other new proteins, as assessed by gel electrophoresis. In three of the experiments (10 Hz, 2 mA/cm<sup>2</sup>; 10 Hz, 4 mA/cm<sup>2</sup> and 1 kHz, 2 mA/cm<sup>2</sup>) cartilage samples near the electrodes in the stimulated chamber (either one or a pair of plugs adjacent to the electrode) synthesized SP70. In two experiments (both at 10 Hz, 2 mA/cm<sup>2</sup>) SP70 was synthesized by plugs not adjacent to the electrode in both experimental and control chambers. In the first of these, two isolated plugs synthesized SP70. In the second, all plugs synthesized SP70.

No significant difference was observed between electrically stimulated and control plugs in total incorporation of <sup>35</sup>S-methionine into proteins that are not extracted in the SDS buffer (e.g. papain

digest radioactivity, Table C.1). Total incorporation of <sup>35</sup>S-methionine into SDS soluble proteins could not be determined for the set of experiments described above. No chase in media with cold methionine was done after labelling, so that free label contributed significantly to the total radioactivity of the gel sample buffer solution. However, free label presumably is removed from the plug by SDS solubilization, and SDS and HBSS washes, so that free label will not contribute significantly to total radioactivity of the papain digest solution, even when no chase is done after labelling.

#### C.4 Discussion and Conclusions

These pilot electrical experiments helped to identify some of the problems with the experimental protocol. Scatter in radioactive counts in aliquots of gel sample buffer solution helped identify the need for a cold methionine chase after labeling. The randomly appearing non-stimulated stress response observed in the early experi-

Frequency	Current density and stimulation protocol	Radioactivity in papain digest (experimental/control ratio)	
		mean	standard deviation
1 Hz	1 mA/cm <sup>2</sup> 12 hour stimulation, then 3 hour label	1.03	0.20
	2 mA/cm <sup>2</sup> , 6 hours	0.98	0.20
10 Hz	2 mA/cm <sup>2</sup> , 6 hours	0.92	0.24
	2 mA/cm <sup>2</sup> , 6 hours	1.04	0.19
	2 mA/cm <sup>2</sup> , 6 hours	1.01	.23
	2 mA/cm <sup>2</sup> , 6 hours then 3 hour label	1.08	0.17
	2 mA/cm <sup>2</sup> 3 hour stimulation then 3 hour label	0.93	0.23
	4 mA/cm <sup>2</sup> , 6 hours	1.03	.23
100 Hz	2 mA/cm <sup>2</sup> , 6 hours	1.25	.41
1 kHz	2 mA/cm <sup>2</sup> , 6 hours	1.19	.21

Table C.1: Total radioactive counts in papain digests for pilot electrical stimulation studies. Radioactive counts, normalized to wet weight are given as the ratio of counts in experimental plugs to their matched controls.

ments may have been due to chamber cleaning that was not complete enough and to insufficient chamber cooling after autoclaving. Randomly appearing stress protein synthesis did not recur after a more careful cleaning and autoclaving protocol was established. SP70 synthesis by plugs adjacent to the electrodes was shown to be due to electrode reaction products (C.2). Thus, random stress protein synthesis identified the need for an especially careful chamber preparation and stress protein synthesis in plugs at the ends of the chambers identified a need to isolate the specimens from the electrodes.

SP70 synthesis in electrical stimulation experiments prior to the changes in protocol, must be regarded with scepticism, including that in which SP70 was synthesized by plugs in the electrically stimulated chamber and not by plugs in the control chamber. There appeared, in fact, to be little reproducible stress response at current densities less than  $2\text{-}4\text{mA}/\text{cm}^2$  at frequencies of 1, 10, 100 Hz, and 1kHz. Papain digests of plugs from these experiments indicated that incorporation of labelled protein into the extracellular matrix was not affected by electrical stimulation at low current densities (at 1, 10, 100 Hz and 1 kHz). Total SDS soluble protein synthesis in earlier experiments could not be determined due to the free label contribution to total gel sample buffer solution radioactivity.

### **C.5 Electrical Response by Plug Position**

The following pages are additional plots of data from some of the electrical experiments which are plotted in terms of the experimental to control ratios of averages of pairs from a single core.

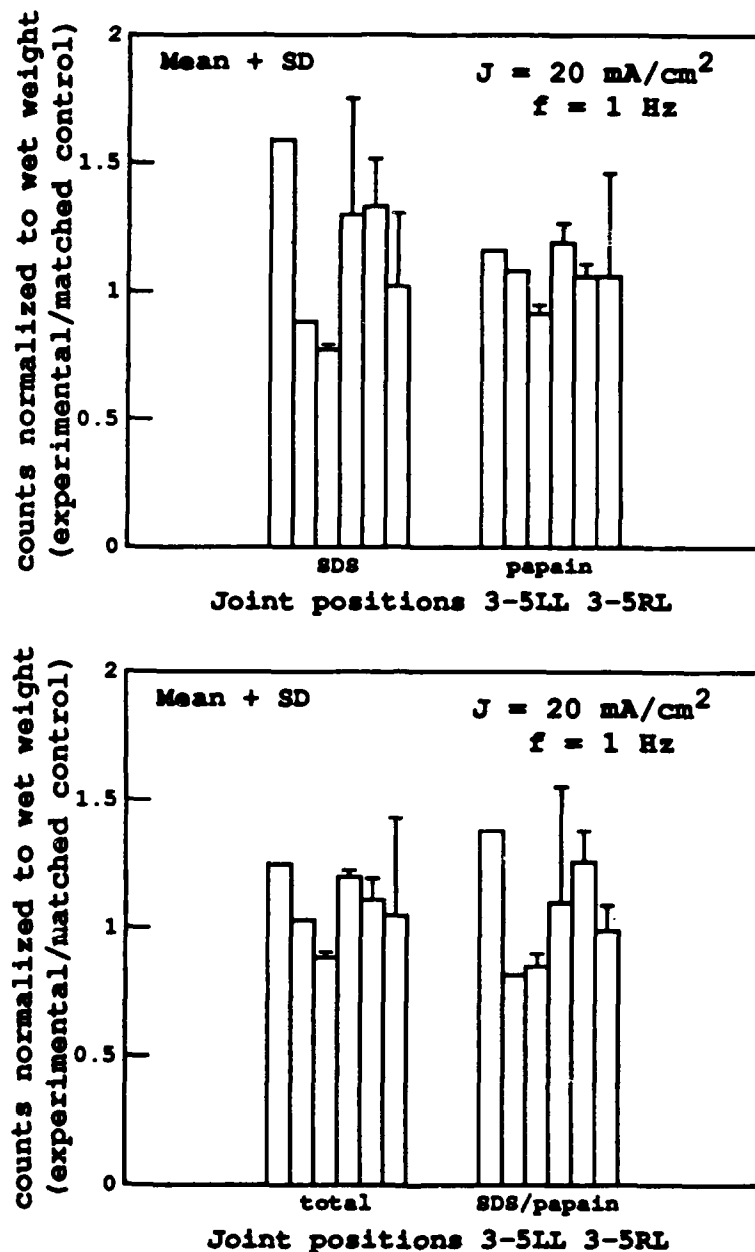


Figure C.1: Electrical response by core position ( $f = 1 \text{ Hz}$ ,  $J = 20 \text{ mA/cm}^2$ ). Experimental/control ratios (cpm normalized to wet weight) are plotted for averages of 2 pairs of matched plugs from a single core. Where no SD bar is shown, the ratio represents data from only 1 pair.

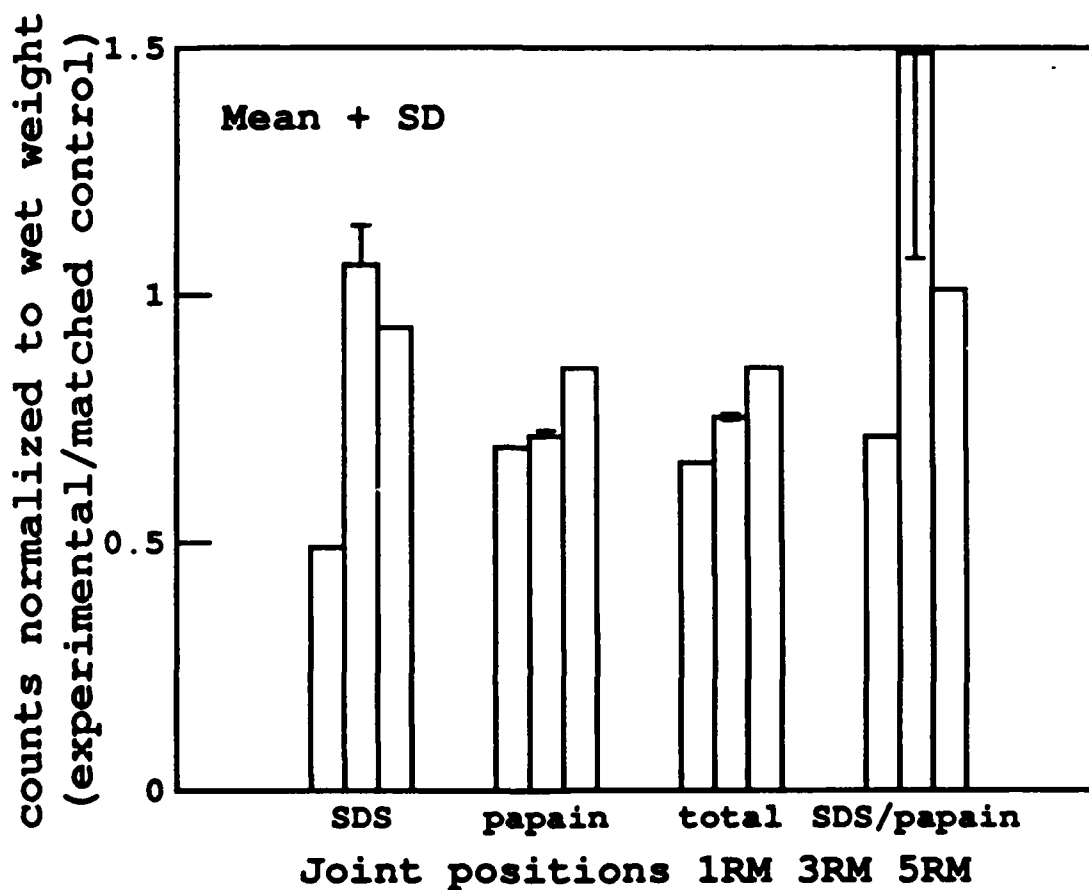


Figure C.2: Electrical response by core position ( $f = 10$  Hz,  $J = 10$  mA/cm<sup>2</sup>). Experimental/control ratios (cpm normalized to wet weight) are plotted for averages of 2 pairs of matched plugs from a single core. Where no SD bar is shown, the ratio represents data from only 1 pair.

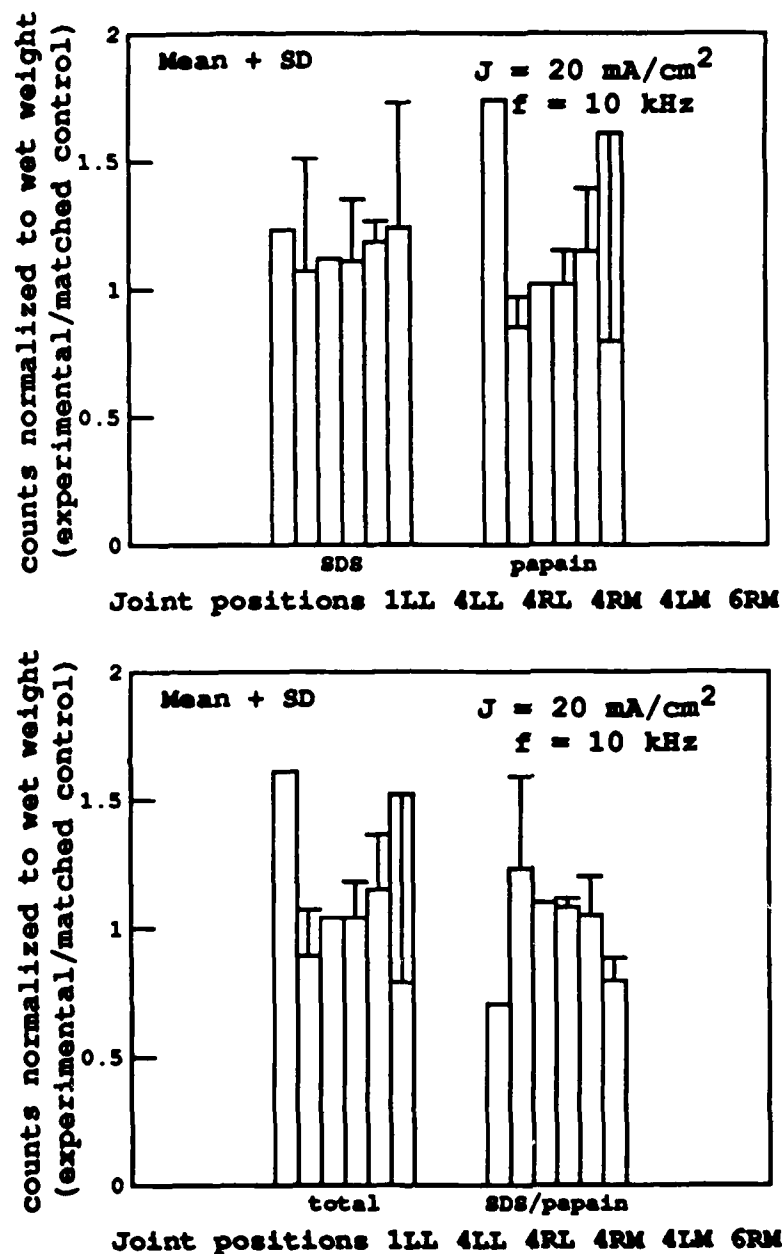


Figure C.3: Electrical response by core position ( $f = 100 \text{ Hz}$ ,  $J = 20 \text{ mA/cm}^2$ ). Experimental/control ratios (cpm normalized to wet weight) are plotted for averages of 2 pairs of matched plugs from a single core. Where no SD bar is shown, the ratio represents data from only 1 pair.

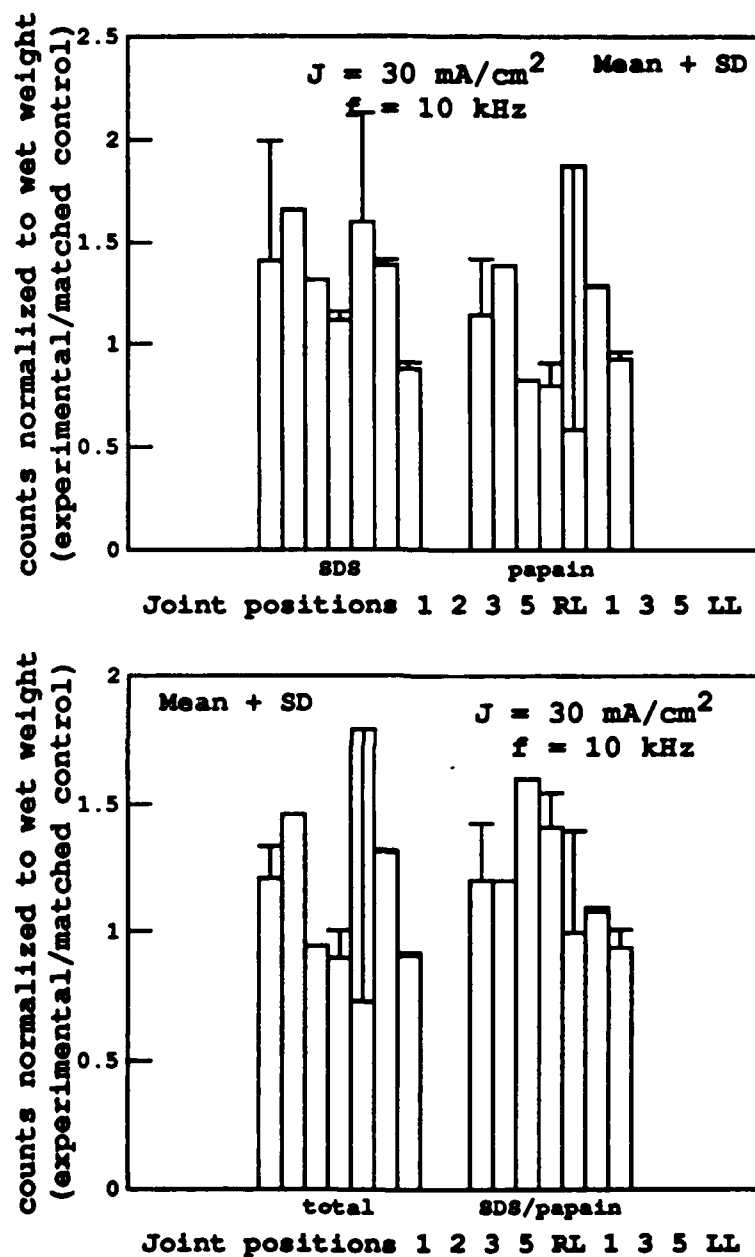


Figure C.4: Electrical response by core position ( $f = 10 \text{ kHz}$ ,  $J = 30 \text{ mA/cm}^2$ ). Experimental/control ratios (cpm normalized to wet weight) are plotted for averages of 2 pairs of matched plugs from a single core. Where no SD bar is shown, the ratio represents data from only 1 pair.



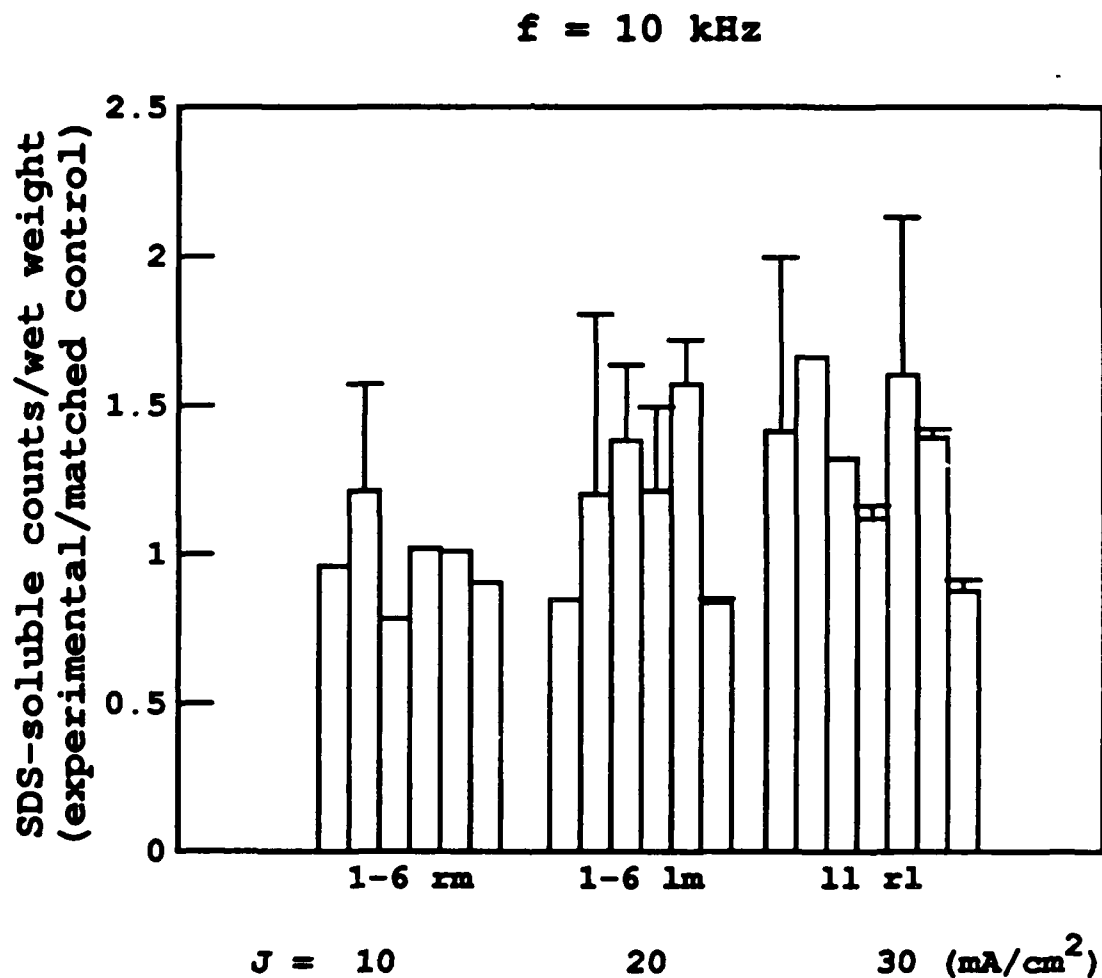


Figure C.5: Electrical response by core position ( $f = 10 \text{ kHz}$ ,  $J = 10, 20$  and  $30 \text{ mA/cm}^2$ ). Experimental/control ratios (cpm normalized to wet weight) are plotted for averages of 2 pairs of matched plugs from a single core. Where no SD bar is shown, the ratio represents data from only 1 pair.

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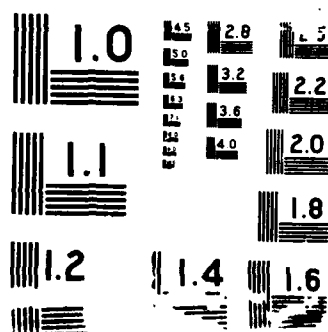
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